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A Comparative Proteomic Analysis of Praziquantel-Susceptible and Praziquantel-Resistant *Schistosoma mansoni* Reveals Distinct Response Between Male and Female Animals

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Schistosomiasis is a chronic neglected tropical disease saddling millions of people in the world, mainly children living in poor rural areas. Praziquantel (PZQ) is currently the only drug used for the treatment and control of this disease. However, the extensive use of this drug has brought concern about the emergence of PZQ-resistance/tolerance by *Schistosoma mansoni*. Studies of *Schistosoma* spp. genome, transcriptome, and proteome are crucial to better understand this situation. In this *in vitro* study, we compare the proteomes of a *S. mansoni* variant strain stably resistant to PZQ and isogenic to its fully susceptible parental counterpart, identifying proteins from male and female adult parasites of PZQ-resistant and PZQ-susceptible strains, exposed and not exposed to PZQ. A total of 60 *Schistosoma* spp. proteins were identified, some of which present or absent in either strain, which may putatively be involved in the PZQ-resistance phenomenon. These proteins were present in adult parasites not exposed to PZQ, but some of them disappeared when these adult parasites were exposed to the drug. Understanding the development of PZQ-resistance in *S. mansoni* is crucial to prolong the efficacy of the current drug and develop markers for monitoring the potential emergence of drug resistance.

Keywords: praziquantel resistance, *Schistosoma mansoni*, proteomics, 2D-electrophoresis, mass spectrometry, liquid chromatography

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INTRODUCTION

Schistosomiasis is one important intravascular parasitic infection, being endemic in 76 countries with more than 97% of infected people living in Africa and parts of South America. This disease infects an estimative of 290 million people worldwide, especially children, living in poor rural areas of low and middle-income countries (1–4). *Schistosoma mansoni* and *S. haematobium* are the two major endemic parasite species causing Schistosomiasis. *S. mansoni* cercariae (shredded by snail hosts) penetrate the human skin and migrate into the vascular system, where mature male and female worms mate and produce ≈ 300 eggs per day (5). *S. mansoni* eggs provoke inflammatory responses and liver damages, which can lead to liver cirrhosis and portal hypertension, as well as an inability to perform a daily activity, headache, fever, syncope, nausea, elevated liver enzymes, malaise, fatigue and nocturnal sweats (4, 5).

Despite many efforts to control the transmission of this disease (6–8), essentially after the introduction of chemotherapeutic treatment in the 1980s, schistosomiasis is still highly prevalent (9). Its control is based on chemotherapy treatment using Praziquantel (PZQ) (10–16), which is effective, inexpensive, and easy to use (10, 16). However, frequent schistosome reinfection occurs, PZQ has minor activity against juvenile parasite forms but has a strong impact on adult worms (10, 17, 18). Because of its high prevalence, schistosomiasis has earned a Category II disease, ranking next to malaria, for importance as a target tropical disease by the World Health Organization special program for research and training in tropical diseases (1–3). Although the impact of schistosomiasis could be dramatically reduced by improvement in education and population awareness, basic sanitation conditions, as well as elimination of the intermediate host snails, such methods are not sufficient to control or eradicate this parasitosis. In the absence of vaccines (4), the control of this disease relies on chemotherapy to ease symptoms and reduce transmission. It is possible to induce resistance of *S. mansoni* and *S. japonicum* to PZQ in mice under laboratorial conditions, and reduced susceptibility to PZQ in the field isolates of *S. mansoni* has been found in many *foci*. Also, there are several schistosomiasis cases caused by *S. haematobium* infections in which repeated standard treatment fails to clear the infection (10, 19, 20). Understanding the development of PZQ-resistance in *S. mansoni* is crucial to prolong the efficacy of the current drug and develop markers for monitoring drug resistance (19, 20).

The identification of proteins is important for understanding how schistosomes regulate host immune systems to establish chronic infections and also elucidate other aspects of parasite–host interaction (21). Furthermore, a comprehensive deciphering of the schistosome genome, transcriptome, and proteome has become increasingly central for understanding the complex parasite–host interplay (12, 22–24). Therefore, such information can be expected to facilitate the discovery of vaccines and new therapeutic drug targets, as well as new diagnostic reagents for schistosomiasis control (21–23), and may aid the development of protein probes for selective and sensitive diagnosis of schistosomiasis (25). Proteomics approaches encompass the most efficient and powerful set of tools for the identification of protein complexes (26–28) and have been widely used to decipher the proteome of parasites such as nematodes (29) and trematodes (30–33). For *Schistosoma* spp., the proteome has been studied in many developmental life stages (34), including lung-schistosomula (35, 36), cercariae (36–38), egg (36, 39) and, adult worms (24, 25, 36, 40–42).

Two-dimensional electrophoresis (2-DE) is a technique for the separation and identification of proteins, based on pI values (isoelectric focusing, IEF) in one dimension and molecular mass by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) in the other (43). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is one of the most commonly used methods to identify and characterize peptides and proteins in complex samples (44). To our knowledge, the *S. mansoni* PZQ-resistant strain proteome has not been yet reported thus an examination of global changes in gene expression following drug treatment or between isolates showing differential drug susceptibility may provide an entrée into the identification of genes underlying drug action or resistance.

Based on the need to identify the genes that underly drug action or resistance, this work aimed to compare the proteomes of a *S. mansoni* variant strain stably resistant to PZQ and isogenic to its fully susceptible parental counterpart, identifying proteins from male and female adult parasites of PZQ-resistant and PZQ-susceptible strains, exposed and not exposed to PZQ. This information represents substantial progress towards deciphering the adult parasite proteome. Furthermore, these data may constitute an informative source for further investigations into PZQ-resistance and increase the possibility of identifying proteins related to this condition, possibly contributing to avoid or decrease the likelihood of development and spread of PZQ-resistance. Improving the understanding of PZQ-resistant and PZQ-susceptible proteome would increase the possibilities of clarification of PZQ mode of action and resistant phenotype.

MATERIAL AND METHODS

Chemical and Reagents

Acetic acid, acetonitrile (ACN), ammonium bicarbonate (NH_4HCO_3), bovine serum albumin (BSA), chloroform, dithiothreitol (DDT), fetal bovine serum (FBS), formic acid (FA), glycerol, iodoacetamide (IAA), isopropyl alcohol, L-glutamine, penicillin–streptomycin solution, phenol, polyacrylamide gel, Praziquantel ($C_{19}H_{24}N_2O_2$), RPMI-1640 medium, sodium dodecyl sulfate (SDS), thiourea, triton X-100, tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), urea, 1,2-diheptanoyl-sn-glycero-3-phosphatidylcholine (DHPC), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich/Merck (Darmstadt, Germany). Agarose and Trizol Invitrogen® reagent were by Thermo Fisher Scientific® (Waltham, Massachusetts,

USA). Complete mini protease inhibitor cocktail tablets were obtained from Roche Diagnostics GmbH® (Mannheim, Germany). Bromophenol blue, Coomassie brilliant blue R-350, IPG buffer, and immobiline dry strip IPG (13 cm; pH 3–10 nonlinear gradient) were obtained from GE Healthcare® (Chicago, IL). Methanol and trifluoracetic acid (TFA) were by Panreac Química S.L.U. ® (Barcelona, Spain). Trypsin modified sequencing grade and trypsin came from Promega Corporation® (Madison, WI). Water was previously distilled and further deionized using a Milli-Q system Millipore (Millipore, Bedford, MA). All solutions were prepared on the day of the experiments.

Ethical Statement

The research study was reviewed and approved by the Ethics Committee and Animal, Faculty of Veterinary Medicine, UTL (Ref. 0421/2013). Animals were maintained and handled following the National and European Legislation (DL 276/2001 and DL 314/2003; 2010/63/EU adopted on September 22, 2010), concerning the protection and animal welfare, and all procedures were performed according to the National and European Legislation. Anesthetics and other techniques were used to reduce the pain and adverse effect on animals.

Parasite Samples

A resistant strain (RS) of *S. mansoni* that tolerates up to 1,200 mg PZQ/kg of mouse body weight (this dose compares to a dose of a single-dose treatment regime of 80 mg/kg in humans) was developed recently (45). As previously described in this strain the female and male parasites phenotypically presented different reactions to PZQ both *in vivo* and *in vitro* (males being more resistant than female parasites). This *S. mansoni* variant strain was selected from a fully susceptible parasite strain, by stepwise drug pressure, and is isogenic, except for the genetic determinants of PZQ-resistance phenotypes, and significantly different from the counterpart *S. mansoni* susceptible strain. As such, this *S. mansoni* PZQ-resistant strain represents a distinct and valuable model for the study of PZQ-resistance.

Thus, two different parasite isolates were used in this study: *S. mansoni* BH PZQ-susceptible strain (SS) from Belo Horizonte (BH), Minas Gerais state, Brazil, and a stable PZQ-resistant strain (RS) (IHMT/UNL) obtained from the same BH strain, as described by Pinto-Almeida et al. (45). These two parasite strains are routinely kept in their intermediate host *Biomphalaria glabrata* snails at the IHMT/UNL (Instituto de Higiene e Medicina Tropical, IHMT; Universidade Nova de Lisboa, UNL, Lisboa, Portugal). *Mus musculus* CD1 line male mice was chosen as the animal model for *S. mansoni* infection because it is a good host for this parasite mimicking the *S. mansoni* human infection (46). Mice infection occurred by natural transdermal penetration of cercariae, by exposing mice tails to about 100 cercariae of *S. mansoni* each.

Eight- to ten-week old adult parasites were recovered by perfusion of the hepatic portal system and mesenteric veins, according to Lewis (47), and washed twice in RPMI-1640, to remove contaminating hair and blood clots. Males and females of *S. mansoni* (resistant strain—RS and susceptible strain—SS to PZQ) were analyzed separately. Regarding the groups of adult parasites exposed to PZQ (EPZQ), after collecting, the parasites were transferred to 24-well culture plates containing RPMI-1640 culture medium, 0.2 mol/L L-glutamine, 0.01 mol/L HEPES, 0.024 mol/L $NaHCO_3$, 10,000 units penicillin, and 10 mg streptomycin/ml, pH 7 and supplemented with 15% FBS. Five adult parasites were added on each well for each studied group for PZQ treatment: 1) PZQ-susceptible male (SM); 2) PZQ-susceptible female (SF); 3) PZQ-resistant male (RM), and 4) PZQ-resistant female (RF). Adult parasites were treated in culture, with 3.0×10^{-5} mol/L of PZQ during 24 h (this dose was chosen and used because it was optimized in our previous published work (45), it allowed us to differentiate between susceptible and resistant parasites in culture, it has an effect on the parasites but that it does not kill them) and, then washed twice with saline solution to clean any traces of culture medium and stored in Trizol at −80°C, for protein extraction later.

For the groups of adult parasites not exposed to PZQ (NEPZQ), adult parasites were kept in RPMI-1640 medium with no addition of drug during 24 h and then washed twice with saline solution to clean any traces of culture medium and, also stored in Trizol at −80°C, for post protein extraction. There are three experimental variables; (1) exposure (E) to Praziquantel (PZQ) or non-exposure (NE), (2) the strain of *S. mansoni*, susceptible (S) or resistant (R), and (3) the sex of the parasite (M/F). Accordingly, the experimental set up consisted of eight sample groups, four for parasites not exposed to PZQ (RM-NEPZQ, RF-NEPZQ, SM-NEPZQ, and SF-NEPZQ), and four for parasites exposed to PZQ (RM-EPZQ, RF-EPZQ, SM-EPZQ, and SF-EPZQ), all experimental groups were done in technical triplicate (2-DE gels were run three times on a single drug treated sample). After the treatment period, the proteins were immediately extracted, all worms were alive prior the protein extraction. In each experimental group proteins were extracted in bulk. The effect of male and female separation on parasite development was not tested *in vitro* in this work.

Preparation of Protein Extracts

S. mansoni adult parasite protein extracts were obtained using Trizol protocol, according to the manufacturer's instructions (48). Briefly, the parasites were lysed and homogenized directly in Trizol reagent at room temperature (≈25°C). The homogenized samples were incubated at room temperature to permit complete dissociation of the nucleoprotein complex. After homogenization, we proceeded to the separation phase, adding chloroform and centrifugation of samples. The aqueous phase was removed and the interphase and organic phenol–chloroform phase was used for the protein isolation procedure. Next, isopropyl alcohol precipitation was performed and the pellet was solubilized in SBI buffer (7 mol/L urea, 2 mol/L thiourea, 0.015 mol/L DHPC, 0.5% Triton X-100, 0.02 mol/L DTT, and complete mini protease inhibitor cocktail tablets), according to Babu et al. (49) and stored at −80°C until use. Protein concentration in protein extracts was measured by Bradford assay (50), and the quality of the extract was verified in 12% uniform SDS-PAGE gels (42).

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis (2-DE) gels was performed in triplicate with 240 μg of protein extracts, for each eight sample group (four not exposed and four parasites exposed to PZQ). To prepare samples for 2-DE, protein samples were diluted in rehydration solution containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 0.5% IPG buffer, 1% DTT, and 0.002% bromophenol blue. The rehydration was carried out passively overnight for 12 h in a 13 cm pH 3–10 strip. The strips were then applied on an Ettan IPGphor 3 System (GE Healthcare, Piscataway, NJ, USA), for protein separation by isoelectric focusing (IEF), using the following conditions developed for this work: at a constant current 50 μA/strip, the voltage program started with a gradient up to 3.5 kV in 3 h, then a step of 3 h at 3.5 kV, then a total voltage of 64.0 kVh to the end.

After focusing, the strips containing proteins were reduced in an equilibration solution (50 mol/L Tris–HCl, pH 8.8, 6 mol/L urea, 20% glycerol, 2% SDS) containing 2% DTT, and then alkylated in the same solution containing 2.5% IAA. The Immobilized pH gradient (IPG) strips and molecular weight standards were then transferred to the top of 12% uniform SDS-PAGE gels, and sealed with 0.5% agarose. The second dimension was carried out using a Protein Plus Dodeca cell system (Bio-Rad Laboratories, Inc., Hercules, CA) under an initial current of 15 mA/gel for 15 min, followed by increasing the current to 50 mA/gel until the end of the run.

For 2-DE experiments, three biological sample replicas were done for the two-dimensional polyacrylamide gel electrophoresis for each group, thus making our experimental procedure more reproducible. Gels were fixed in 40% methanol/10% acetic acid solution and stained with coomassie brilliant blue R-350. The spots were visually normalized and evaluated using the ImageMaster® 2D Platinum 7.0 software (GE Healthcare Bio-Sciences, Uppsala, Sweden), according to the number of spots matching, and the results were exported and evaluated in SIMCA-P software (Umetrics, Umeå, Sweden) as previously described. Here, we built PCA models, from which it was possible to obtain statistically valid spots between groups using *t*-test and Jackknife. This was done in a way that the software would inform us on the proteins spots of interest (51).

In-Gel Digestion and Peptide Preparation for Mass Spectrometry Analysis

We selected spots that were consistently found in all replicates for each condition, and were large enough to excise and retain sufficient material for downstream processing. The protein spots selected for each group were manually excised, destained, reduced, alkylated, and digested in-gel with trypsin from the corresponding 2-DE gel for mass spectrometry (MS) identification. First, spots were washed in ultrapure water and then destained in a solution containing 50% methanol/2.5% acetic acid, for 2 h at room temperature. This step was repeated until clear of blue stain. The gel fragments were incubated in 100% ACN with occasional vortexing until gel pieces became white and shrank. Then, the solution was removed and spots were completely dried and ready for digestion. The in-gel digestion with trypsin modified sequencing grade reagents was done according to Shevchenko et al. (52). Briefly, protein digestion was conducted at 37°C overnight. After the incubation, the supernatant was transferred to a clean tube, and 30 μl of 5% FA/60% ACN solution was added to gel spots for the extraction of tryptic peptides. This procedure was performed two times for 30 min, under constant agitation. The supernatant was pooled to the respective tube containing the initial peptide solution. This solution was dried in a Speedvac concentrator Thermo Fisher Scientific (Waltham, MA) and the peptides were re-suspended in 8 μl of 0.1% FA solution. The peptides were desalted in reverse phase micro-columns ZipTip® C18 (Millipore Corporation, Billerica, MA), according to the manufacturer's instructions (53). Peptides were dried again and resuspended in 50% ACN/0.1% TFA solution.

Nanoflow LC-MS/MS Analysis and Protein Identification

The digested peptides (50 μg) were analyzed in an Easy-nLC II nanoflow liquid chromatography system (nano-LC-MS/MS) (Thermo Fisher Scientific, Waltham, MA) in tandem with an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific), which was equipped with a nanoelectrospray Nano-Flex II (Thermo Fisher Scientific) operating in a positive ion mode (24). Nanoelectrospray voltage was set to 4.5 kV and source temperature at 220°C. The precursor ion was isolated using the data-dependent acquisition mode with a 2 *m/z* isolation width and sequentially the ten most intense ions were selected for fragmentation event by collision-induced dissociation (CID), at 35% normalized collision energy and 10 ms activation time. Maximum ion injection times were set to 100 ms for MS and 500 ms for MS/MS, with a resolution of 60,000 and a scan within *m/z* range from 400 to 2,000. Chromatographic separation was carried out a Thermo C18 capillary column (10 cm × 75 μm, 3 μm, 120 Å) protected by a guard Thermo C18 capillary column (2 cm × 100 μm i.d., 5 μm particle, 120 Å pore size). Ultrapure water containing 0.1% FA (solvent A) and ACN containing 0.1% FA (solvent B) was used as mobile phase. The separation was performed at room temperature with a constant flow rate of 0.3 μl/min, with a total acquisition time of 90 min, by employing the elution program as follows: linear gradient of 5% of solvent B during 5 min, ranging to 80% of solvent B over 85 min. Data acquisition was controlled by Xcalibur® 2.0.7 Software (Thermo Fisher Scientific) and converted in mgf files using MassMatrix® MS Data file conversion version 3.9 software.

Bioinformatics

The list of peptide and fragment mass values generated by the mass spectrometer for each spot were submitted to an MS/MS ion search using the Mascot® 2.0 software online search engine (Matrix Science Inc, Boston, MA, USA) to search the Nanoflow LC-MS/MS data against the NCBInr database *Schistosoma_mansoni*_NCBI_112014, November 2014. Mascot® software (used at the Mass Spectrometry Laboratory at Brazilian Biosciences National Laboratory, CNPEM, Campinas, Brazil) and also against WormBase ParaSite, was set with

two tryptic missed cleavages, a peptide ion mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.02 Da, a peptide charge state of 2+ and 3+, variable modifications of methionine (oxidation), and fixed modifications of cysteine (carbamidomethylation).

During the analysis, our samples were checked against a contaminant database supplied by the Global Proteome Machine [GPM-web-based, an open-source user interface for analyzing and displaying protein identification data by Craig and Beavis (54)]. All validated proteins had at least two independent spectra, with greater than 95.0% probability estimated by the Peptide Prophet algorithm of being present in the *S. mansoni* database as at least one unique peptide. To avoid random matches, only ions with an individual score above the indicated by the Mascot® to identity or extensive homology (p <0.05) were considered for protein identification. However, when the Mascot® score was not significant, but the percentage coverage and root mean squared error (RMSE) were in the same range as those of proteins with a significant match, proteins were deemed identified if additional parameters, such as its calculated p*I* and *M*w, were in agreement with those observed for the actual gel spot and the species matched was *S. mansoni*.

The molecular function and biological process were assigned for the proteins identified according to information obtained from the Gene Ontology® (GO) database (55). The exact annotation for each protein was used in most cases. However, the catalytic activity category was used for all proteins with molecular functions associated with (GTPase, hydrolase, isomerase, kinase, ligase, lyase, oxidoreductase, transcription, and transferase activities). Binding category was used for all types of ligand identified (actin, ATP, calcium, GTP, magnesium ion, metal ion, protein domain-specific, and nucleotide bindings). Furthermore, there were other molecular function categories classified such as chaperone, motor, regulation of muscle contraction, structural, and transport. The proteins that had no associated known function were classified as "unknown".

The list of proteins that were identified in all the experiments was analysed using the webserver g:Profiler, to perform functional enrichment analysis. Briefly, the g:GOSt functionality was used, considering a g:SCS threshold <0.05 for statistical significance (56).

RESULTS

2-DE Separation of Proteins From *S. mansoni* PZQ-Resistant and PZQ-Susceptible Adult Parasites

Eight protein extracts were analyzed: four from parasites not exposed to PZQ (NEPZQ) [resistant males and females (assigned as RM-NEPZQ and RF-NEPZQ, respectively), and susceptible males and females (assigned as SM-NEPZQ and SF-NEPZQ, respectively)] and another four samples from parasites exposed to PZQ (EPZQ) [resistant males and females (assigned as RM-EPZQ and RF-EPZQ, respectively), and susceptible males and females (assigned as SM-EPZQ and SF-EPZQ, respectively)]. All protein extracts presented high purity and good quality for posterior 2-DE (**Figure 1**) and nano-LC-MS/MS analysis. The quality/purity of the protein extract was assessed as previously described by Rayna et al. (57), the quality/purity of the extract was verified in 12% uniform SDS-PAGE gels.

Analytical 2-DE gels were produced, to reproducibly resolve protein spots in a broad pH range and molecular weight, and to compare the protein pattern of *S. mansoni* proteome from the two strains (PZQ-resistant and PZQ-susceptible) not exposed (**Figure 2**) and exposed to PZQ (**Figure 3**). See **Figures 2**, **3**, and **Supplementary Data**.

Two-DE maps constructed with coomassie blue-stained gels showed reasonably comparable numbers of spots in all the samples. In total 133 ± 14, 265 ± 20, 142 ± 8, and 188 ± 34 spots were detected in proteins from RM-NEPZQ, RF-NEPZQ, SM-NEPZQ, and SF-NEPZQ, respectively (**Table 1**). For parasites exposed to PZQ, 203 ± 14, 133 ± 9, 220 ± 34, and 99 ± 19 spots were detected in RM-EPZQ, RF-EPZQ, SM-EPZQ, and SF-EPZQ, respectively (**Table 1**). **Table 1** includes the data from the replicates. The spots that were validated were the same in each replicate. The only protein spots whose analysis was continued, were those where a protein spot is consistently expressed in the three replicates, if the protein spot is only expressed in one or two replicas then this spot is excluded from the subsequent analysis.

Nanoflow LC-MS/MS Analysis and Protein Identification

Gels were compared between replica and the only spots that were chosen to be excised were the ones that were more intense and that were present in all three replicas. The spots were excised from preparative gels for each sample, digested by trypsin, and identified by nanoflow LC-MS/MS. For RM-NEPZQ, 64 spots were successfully analyzed by nano-LC-MS/MS, as well as 69 from RF-NEPZQ, 67 from SM-NEPZQ, 68 from SF-NEPZQ, 68 from RM-EPZQ, 69 from RF-EPZQ, 67 from SM-EPZQ, and 66 spots from SF-EPZQ (**Supplementary Material Tables 1–3**). The nano-LC-MS/MS results were employed to search the database (NCBInr) by the Mascot® search engine, and the matched proteins are listed in **Supplementary Tables 1, 2**. Some proteins were identified in only one individual spot, but on several occasions, more than one spot in a gel corresponded to the same protein, or better, same protein species (**Figures 2**, **3**, and **Supplementary Material Tables 1–3**).

Sixty individual protein species were identified on samples from parasites not exposed to PZQ, of which 45 were present in RM-NEPZQ, 52 in RF-NEPZQ, 45 in SM-NEPZQ, and 47 proteins in SF-NEPZQ (**Supplementary Material**). In this group of NEPZQ parasites, 35 proteins were common to all four protein extracts, eight occurred only in resistant adult parasites, eight only in susceptible adult parasites, four in female parasites, and three were only present in resistant females (**Figure 4**). Interestingly, two proteins that have shown to be common in RM-NEPZQ, RF-NEPZQ, and SM-NEPZQ preparations, namely serine/threonine phosphatase and troponin I, did not appear in SF-NEPZQ (**Table 2**).

The total number of proteins identified was reduced to 55 proteins on the protein extracts from parasites exposed to PZQ.

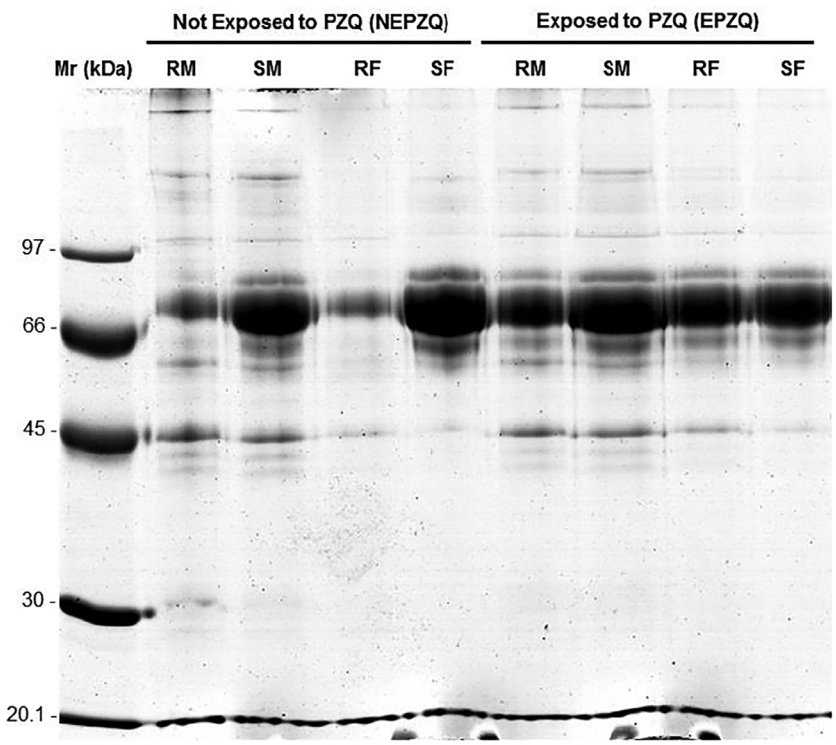

FIGURE 1 | SDS-PAGE gel of the protein preparations in all eight conditions, improving the quality of the protein extracts studied. RM, resistant males; SM, susceptible males; RF, resistant females; SF, susceptible females; Mr, Molecular marker reference.

Forty-eight of those proteins were present in RM-EPZQ, 52 in RF-EPZQ, 45 in SM-EPZQ, and 46 in SF-EPZQ (**Supplementary Material Tables 1–3**). Forty-two proteins appeared to be common to all the four protein extracts of EPZQ parasites. Besides, four proteins were present only in resistant strain, one in susceptible strain, three were exclusive of female parasites, two proteins were present only in male parasites, and three were only in resistant females (**Figure 4**). Five proteins that were present on parasites not exposed to PZQ, namely, collagen alpha chain, dopamine transporter, twister (putative), ubiquitin-specific peptidase 30 (C19 family), and Smp_162220, did not appear here (**Table 2**).

Molecular Function of Identified Proteins

The proteins identified by nano-LC-MS/MS, summarized in **Supplementary Tables 1, 2**, were generally categorized by their molecular function, according to information obtained from the GO database (**Table 3**), we consider that this general classification in large groups of protein functions is fundamental as a basis for future proteomics and transcriptomic tests. When proteins had another function annotation, they were shown in brackets. The biological process and subcellular localization assigned to each protein in that database are also included in **Table 3**. The results allowed the identification of proteins categorized as binding, catalytic, transport, regulation of muscle contraction, chaperone, motor, structural activities, and proteins of unknown functions. Among the molecules identified as binding proteins, most of them were ATP, nucleotide, protein, and ion binding proteins. The proteins categorized correspond to a variety of biological processes, nevertheless, most of them were glycolytic enzymes and proteins related to the metabolic process.

Regarding the subcellular localization, the proteins identified were classified, as cytoskeletal, cytosolic, nuclear, membrane proteins, and some of them were located on extracellular matrix. Among them, those most abundantly identified were cytosolic proteins. There were fifteen and seven proteins whose biological process and subcellular localization, respectively, were not predicted (**Table 3**).

Functional Enrichment Analysis

In all experimental conditions, except in both exposed or not exposed susceptible females, the GO Molecular Processes with the highest p-values were related to muscle contraction (GO:0006936, GO:0003012, GO:0003008), corresponding to UniProt geneIDs P42637, P42638, G4VGQ7, G4LYF7. However, in susceptible females, both exposed and unexposed, the Molecular Processes with the highest p-values were unique and related to ADP metabolic process and ATP generation from ADP (GO:0046031, GO:0006757, GO:0009135, GO:0006096, GO:0009179, GO:0009185, GO:0006165, GO:0046939,

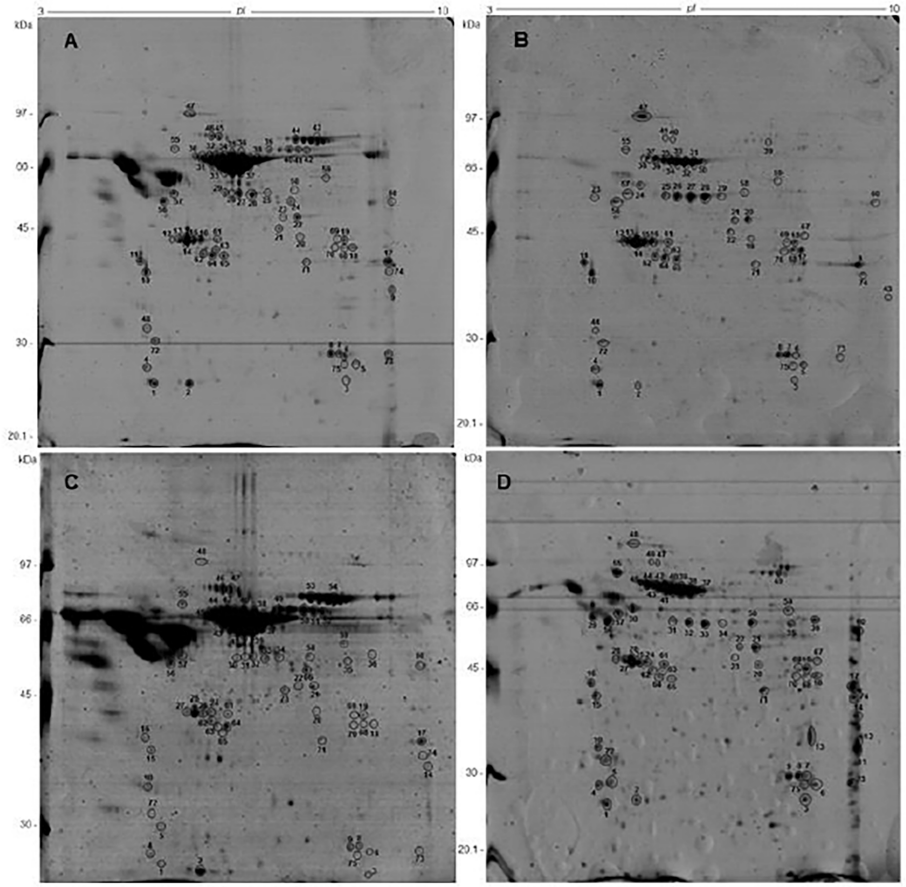

FIGURE 2 | Comparative two-dimensional gel electrophoresis of protein samples from *S. mansoni* adult parasites not exposed to PZQ (baseline experiment), using 13 cm, pH 3-10NL strips and SDS-PAGE 12%, stained by coomassie brilliant blue: **(A)** SM-NEPZQ; **(B)** RM-NEPZQ; **(C)** SF-NEPZQ; **(D)** RF-NEPZQ. The identified spots, which were analyzed and identified by Nanoflow LC-MS/MS. All the identified proteins are listed in **Supplementary Material Tables 1–3**.

GO:0006090, and GO:0009132). These GO terms were associated with gene IDs P53442, P20287, G4VJD5, and G4VQ58 (**Table 4**) (**Supplementary Tables 1, 3**).

DISCUSSION

Nowadays the control of schistosomiasis is based on PZQ, which is the only drug available for its treatment. The WHO strategy for schistosomiasis control focuses on reducing disease through periodic, targeted treatment with PZQ through the large-scale treatment of affected populations. It involves regular treatment of all at-risk groups (6, 8, 12, 16). However, the existence of reports of PZQ-resistance cases by *S. mansoni* (as well as by *S. haematobium*) has become a serious problem that needs to be solved. Some studies have suggested that resistance to *Schistosoma* infection can be acquired naturally or induced by the drug (20, 45, 58–60). For example, Sanchez et al. (20) examined the effect of PZQ in strains of *S. mansoni*. The authors noted a reduced miracidia susceptibility after exposure to 2 × 50 mg/Kg of PZQ. It has also been reported the susceptibility of worms *in vivo* (mice infected with schistosomes) after a lethal dose of 3 × 300 mg/kg of PZQ, reducing in only 10% the worm number in infected rodents. Besides experimental evidence, reports of treatment failure in Senegal and Egypt with reduced susceptibility to PZQ were obtained (61, 62) and, further *in vitro* experiments have confirmed the development of PZQ-resistance (9, 10, 43, 61–64).

In the present study we identified 60 different proteins on *S. mansoni* proteome, this corresponds to 0.42% of the full *S. mansoni* proteome (https://www.uniprot.org/proteomes/UP000008854). All those proteins were present in adult parasites not exposed to PZQ, but some of them disappeared when these adult parasites were exposed to the drug. This result could indicate the involvement of PZQ exposure on those protein expressions in resistant and susceptible strains. With this, we are not unequivocally stating that these proteins are the only ones or that they are at all responsible for the phenotype of PZQ drug resistance but this work opens the door to new studies using proteomics, transcriptomics, and metabolomics on the

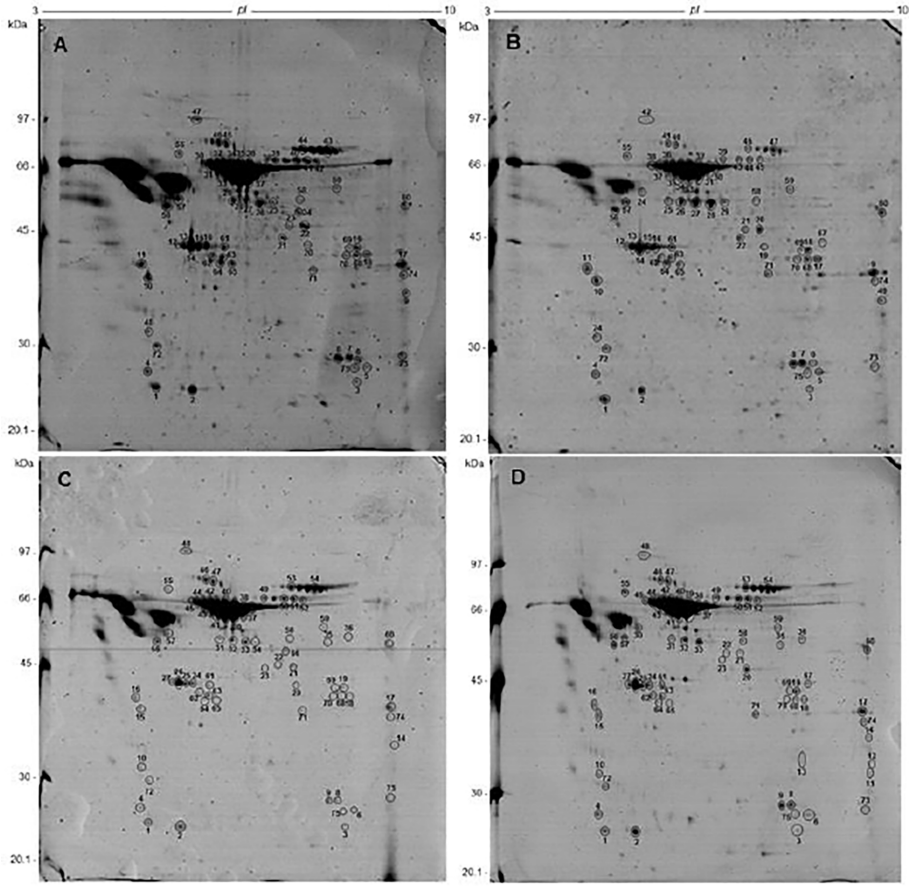

FIGURE 3 | Comparative two-dimensional gel electrophoresis of protein samples from *S. mansoni* adult parasites exposed to PZQ using 13 cm, pH 3-10NL strips and SDS-PAGE 12%, stained by coomassie brilliant blue. **(A)** SM-EPZQ; **(B)** RM-EPZQ; **(C)** SF-EPZQ; **(D)** RF-EPZQ. The identified spots, which were analyzed and identified by Nanoflow LC-MS/MS. All the identified proteins are listed in **Supplementary Material Tables 1–3**.

TABLE 1 | Summary comparison of the number of protein spots in the 2-DE maps for the eight different proteome samples analyzed.

	Not exposed to PZQ (NEPZQ)				Exposed to PZQ (EPZQ)			
	RM	***RF***	***SM***	***SF***	***RM***	***RF***	***SM***	***SF***
Replica 1	145	243	151	150	215	124	242	80
Replica 2	135	270	138	200	188	135	181	99
Replica 3	118	281	136	215	207	141	237	117
Mean ± SD[a]	**133 ± 14**	**265 ± 20**	**142 ± 8**	**188 ± 34**	**203 ± 14**	**133 ± 9**	**220 ± 34**	**99 ± 19**

[a]*SD, standard deviation.*

The only protein spots whose analysis was continued, were those that where a protein spot is consistently expressed in the three replicates, if the protein spot is only expressed in one or two replicas then this spot is excluded from the subsequent analysis.

In bold Mean ± SD.

importance of these proteins in the phenotype and genotype of resistance to PZQ.

Although previous studies of *Schistosoma* proteome had been performed using protein extracts and *Schistosoma* species different from ours, some proteins, such as 14-3-3 protein, HSP-70, GAPDH, glutathione S-transferase 28 kDa, enolase, fructose-bisphosphate aldolase, actin, triosephosphate isomerase, calponin, elongation factor 1-α, phosphoglycerate kinase, phosphoglycerate mutase, myosin, and paramyosin were commonly identified (36, 42, 65–70). In addition, some proteins that have already been tested as vaccine candidates, as glutathione S-transferase 28 kDa (71), triosephosphate isomerase (71, 72), and paramyosin (71), were also identified in the present study. Major egg antigen, troponin T, disulphide-isomerase ER-

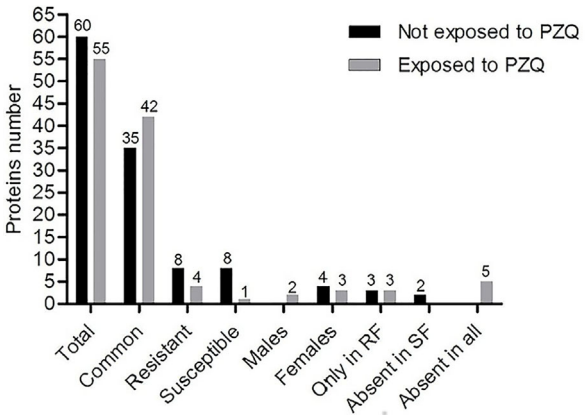

FIGURE 4 | Number of shared proteins identified from parasites not exposed and exposed to Praziquantel.

60, and actin, proteins that we also found, have already been clustered as immunoreactive proteins in serum pools of infected or non-infected individuals from endemic area (42).

Looking at the proteomes from both genders, in this survey, four proteins were only present in females from both strains, even under exposure to PZQ, namely, cytosol aminopeptidase, inosine-5′-monophosphate dehydrogenase (IMPDH), ubiquitin protein ligase E3a, and collagen alpha chain. Cytosol/leucine aminopeptidase catalyzes the hydrolysis of amino acid residues from the N-terminus of proteins and peptides (73), and it has already been assessed as a vaccine candidate against the infection of *Fasciola hepatica* (74). This protein has previously been identified in *S. mansoni* eggs (75). Regarding IMPDH, this protein is responsible for the rate-limiting step in guanine nucleotide biosynthesis (76), and it has previously been identified in the *Schistosoma* genome and transcriptome (77). E3 ligase enzyme catalyzes protein ubiquitination, which regulates various biological processes through covalent modification of proteins and transcription factors, and ubiquitin is the most important protein of this process (78, 79). Ubiquitination is of interest in *S. mansoni* because this process could be a potential target for the design of new drugs (80), being ubiquitin protein ligase E3a a good target to be studied. Concerning collagen alpha chain, Yang et al. (81) described that silencing the expression of a type of collagen (type V collagen) significantly affects the spawning and egg hatching of *S. japonicum*, and it also affects the morphology of the adult parasites (81). Therefore, it would be very interesting to evaluate the role of each of these proteins in PZQ-resistance, especially, collagen alpha chain, since it seems to produce morphological alterations in eggs and adult parasites of *S. mansoni* PZQ-resistant strain (10, 11). Moreover, females of this species, the only gender in which those proteins were found in this work, are more tolerant to PZQ treatment than males (45).

TABLE 2 | Summary comparison of the specific proteins identified from parasites not exposed (NEPZQ) and exposed (EPZQ) to Praziquantel.

Proteins	Not exposed to PZQ				Exposed to PZQ			
	SM	RM	SF	RF	SM	RM	SF	RF
ATP-dependent transporter	Yes	–	Yes	–	Yes	Yes	Yes	Yes
Beta1,3-galactosyltransferase	–	–	–	Yes	–	–	–	Yes
Cathepsin L	–	–	–	Yes	–	–	–	Yes
Collagen alpha chain	–	–	Yes	Yes	–	–	–	–
Cytosol aminopeptidase	–	–	Yes	Yes	–	–	Yes	Yes
Dopamine transporter	–	Yes	–	Yes	–	–	–	–
Galactokinase	–	Yes	–	Yes	–	Yes	–	Yes
Inosine-5'-monophosphate dehydrogenase	–	–	Yes	Yes	–	–	Yes	Yes
Lysine tRNA ligase	Yes	–	Yes	–	Yes	Yes	Yes	Yes
Ornithine aminotransferase	–	Yes	–	Yes	–	Yes	–	Yes
Phosphopyruvate hydratase	Yes	–	Yes	–	Yes	–	Yes	–
Protein kinase	Yes	–	Yes	–	Yes	Yes	Yes	Yes
Rab6-interacting protein 2 (ERC protein 1)	–	Yes	–	Yes	–	Yes	–	Yes
Receptor for activated Protein Kinase C (PKC)	–	–	–	Yes	–	–	–	Yes
RNA m5u methyltransferase	Yes	–	Yes	–	Yes	Yes	Yes	Yes
Serine/threonine phosphatase	Yes	Yes	–	Yes	Yes	Yes	–	–
Suppressor of actin (Sac)	Yes	–	Yes	–	Yes	Yes	Yes	Yes
Transducin beta-like	-	Yes	–	Yes	–	Yes	–	Yes
Troponin I	Yes	Yes	–	Yes	Yes	Yes	–	–
Twister (putative)	–	Yes	–	Yes	–	–	–	–
Ubiquitin protein ligase E3a	–	–	Yes	Yes	–	–	Yes	Yes
Ubiquitin-specific peptidase 30 (C19 family)	–	Yes	–	Yes	–	–	–	–
Uncharacterized protein, Smp_018790 (91.6% identity with PP2C-like domain-containing protein - *S. haematobium*)	Yes	–	Yes	–	Yes	Yes	Yes	Yes
Uncharacterized protein, Smp_161260 (63.4% identity with SJCHGC05745 protein—*S. japonicum*)	Yes	–	Yes	–	Yes	Yes	Yes	Yes
Uncharacterized protein, Smp_162220 (88% identity with SJCHGC07938 protein—*S. japonicum*)	–	Yes	–	Yes	–	–	–	–

SM, susceptible male; RM, resistant male; SF, susceptible female; RF, resistant female; Yes, protein expressed; –, protein not expressed.

TABLE 3 | Proteins identified by MS/MS and categorized by their molecular function according to information obtained from GO database.

	Term name	Accession n°	Protein	Exposed to PZQ	Not Exposed to PZQ
1	Actin cytoskeleton	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
2	Actin filament	P42637	Tropomyosin-1	Yes	Yes
3	ADP metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
4	ATP generation from ADP	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
5	Biosynthesis of amino acids	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	No	Yes
6	Carbohydrate catabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
7	Carbon metabolism	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VBJ0	Malate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	No	Yes
8	Carboxylic acid metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4M0E0	Lysine tRNA ligase	Yes	Yes
		G4VBJ0	Malate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
9	Contractile fiber	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
10	Cytoskeleton	P53470	Actin-1	Yes	Yes
		Q26595	Alpha tubulin	Yes	Yes
		P14202	Tegument antigen (Antigen SmA22.6)	Yes	Yes
		P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
		C4QIC0	Tubulin beta chain	Yes	Yes
11	Generation of precursor metabolites and energy	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VBJ0	Malate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
12	Glycolysis / Gluconeogenesis	G4LWI3	Aldehyde dehydrogenase	Yes	Yes
		P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	No	Yes
13	Glycolytic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
14	Monocarboxylic acid metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
15	Muscle contraction	P42637	Tropomyosin-1	Yes	Yes

(Continued)

TABLE 3 | Continued

	Term name	Accession n°	Protein	Exposed to PZQ	Not Exposed to PZQ
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
16	Muscle system process	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
17	Muscle thin filament tropomyosin	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
18	Myofibril	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
19	Myofilament	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
20	Nucleoside diphosphate metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
21	Nucleoside diphosphate phosphorylation	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
22	Nucleotide phosphorylation	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
23	Organic acid metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4M0E0	Lysine tRNA ligase	Yes	Yes
		G4VBJ0	Malate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
24	Oxoacid metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4M0E0	Lysine tRNA ligase	Yes	Yes
		G4VBJ0	Malate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
25	Polymeric cytoskeletal fiber	Q26595	Alpha tubulin	Yes	Yes
		P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		C4QIC0	Tubulin beta chain	Yes	Yes
26	Purine nucleoside diphosphate metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
27	Purine ribonucleoside diphosphate metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
28	Purine ribonucleotide metabolic process	P53442	Fructose bisphosphate aldolase	No	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	No	Yes
		G4V9I5	Inosine-5'-monophosphate dehydrogenase	No	Yes
		G4VJD5	Phosphoglycerate mutase	No	Yes
		G4VQ58	Phosphopyruvate hydratase	No	Yes
29	Purine-containing compound metabolic process	P53442	Fructose bisphosphate aldolase	No	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	No	Yes
		G4V9I5	Inosine-5'-monophosphate dehydrogenase	No	Yes
		G4VJD5	Phosphoglycerate mutase	No	Yes
		G4VQ58	Phosphopyruvate hydratase	No	Yes

(Continued)

TABLE 3 | Continued

	Term name	Accession n°	Protein	Exposed to PZQ	Not Exposed to PZQ
30	Pyruvate metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
31	Ribonucleoside diphosphate metabolic process	P53442	Fructose bisphosphate aldolase	Yes	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	Yes	Yes
		G4VJD5	Phosphoglycerate mutase	Yes	Yes
		G4VQ58	Phosphopyruvate hydratase	Yes	Yes
32	Ribonucleotide metabolic process	P53442	Fructose bisphosphate aldolase	No	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	No	Yes
		G4V9I5	Inosine-5'-monophosphate dehydrogenase	No	Yes
		G4VJD5	Phosphoglycerate mutase	No	Yes
		G4VQ58	Phosphopyruvate hydratase	No	Yes
33	Ribose phosphate metabolic process	P53442	Fructose bisphosphate aldolase	No	Yes
		P20287	Glyceraldehyde-3-phosphate dehydrogenase	No	Yes
		G4V9I5	Inosine-5'-monophosphate dehydrogenase	No	Yes
		G4VJD5	Phosphoglycerate mutase	No	Yes
		G4VQ58	Phosphopyruvate hydratase	No	Yes
34	Sarcomere	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
35	Striated muscle thin filament	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
36	Structural constituent of muscle	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
37	Supramolecular complex	Q26595	Alpha tubulin	Yes	Yes
		P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
		C4QIC0	Tubulin beta chain	Yes	Yes
38	Supramolecular fiber	Q26595	Alpha tubulin	Yes	Yes
		P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
		C4QIC0	Tubulin beta chain	Yes	Yes
39	Supramolecular polymer	Q26595	Alpha tubulin	Yes	Yes
		P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
		C4QIC0	Tubulin beta chain	Yes	Yes
40	System process	P42637	Tropomyosin-1	Yes	Yes
		P42638	Tropomyosin-2	Yes	Yes
		G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes
41	Troponin complex	G4VGQ7	Troponin I	Yes	Yes
		G4LYF7	Troponin T	Yes	Yes

Another large difference between the proteome of both genders was the expression of troponin I and serine/threonine-protein phosphatase. These proteins were present in males independently of drug exposure, but in females, they were only present in resistant females not exposed to PZQ. Troponin I belongs to the troponin complex that mediates Ca^{2+} regulation that governs the actin-activated myosin motor function in striated muscle contraction (82). On the other side, protein kinases and phosphatases, as is the case of serine/threonine-protein phosphatase, are essential for the normal functioning of signaling pathways since it is well known that reversible phosphorylation of proteins is a ubiquitous mechanism crucial for the regulation of most cellular functions (83). In *S. mansoni*, a phosphatase 2B (calcineurin) has been described as a heterodimer with a catalytic subunit and a regulatory subunit, which bind to Ca^{2+} increased the phosphatase activity (84). Thus, protein phosphatases represent crucial molecules for the parasite and hence potential chemotherapeutic targets (85).

TABLE 4 | Summary comparison of the specific proteins identified from parasites not exposed (NEPZQ) and exposed (EPZQ) to Praziquantel on gene ontology analyses.

		Exposed				Non Exposed			
term_name	**term_id**	**Resistant Females**	**Resistant Males**	**Susceptible Females**	**Susceptible Males**	**Resistant Females**	**Resistant Males**	**Susceptible Females**	**Susceptible Males**
		p_value	**p_value**	**p_value**	**p_value**	**p_value**	**p_value**	**p_value**	**p_value**
Structural constituent of muscle	GO:0008307		0.049870		0.049910	0.049916	0.033353		0.04991
muscle system process	GO:0003012	**0.003688**	**0.000010**	**0.002188**	**0.000010**	**0.000017**	**0.000010**	**0.00218**	**0.00001**
muscle contraction	GO:0006936	**0.003688**	**0.000010**	**0.002188**	**0.000010**	**0.000017**	**0.000010**	**0.00218**	**0.00001**
glycolytic process	GO:0006096	0.018497	0.010990	**0.000121**	**0.000106**	0.014460	**0.009647**	**0.00012**	**0.00010**
ATP generation from ADP	GO:0006757	0.018497	0.010990	**0.000121**	**0.000106**	0.014460	**0.009647**	**0.00012**	**0.00010**
purine ribonucleoside diphosphate metabolic process	GO:0009179	0.018497	0.010990	**0.000121**	**0.00106**	0.014460	**0.009647**	**0.00012**	**0.00010**
purine ribonucleotide metabolic process	GO:0009150			**0.004901**					
ribonucleotide metabolic process	GO:0009259			**0.008112**					
purine-containing compound metabolic process	GO:0072521			0.010564					
ribose phosphate metabolic process	GO:0019693			**0.008674**		0.014460	**0.009647**	**0.00012**	**0.00010**
purine nucleoside diphosphate metabolic process	GO:0009135	0.018497	0.010990	**0.000121**	**0.000106**	0.014460	**0.009647**	**0.00012**	**0.00010**
ribonucleoside diphosphate metabolic process	GO:0009185	0.018497	0.010990	**0.000121**	**0.000564**	0.014460	**0.009647**	**0.00012**	**0.00010**
ADP metabolic process	GO:0046031	0.018497	0.010990	**0.000121**	**0.000106**	0.033912	0.022665	**0.00039**	**0.00034**
nucleoside diphosphate phosphorylation	GO:0006165	0.043351	0.025790	**0.000396**	**0.000348**	0.033912	0.022665	**0.00039**	**0.00034**
nucleotide phosphorylation	GO:0046939	0.043351	0.025790	**0.000396**	**0.000348**	0.047993	0.032107	**0.00064**	**0.00056**
pyruvate metabolic process	GO:0006090		0.036510	**0.000640**	**0.000564**	0.047993	0.032107	**0.00064**	**0.00056**
nucleoside diphosphate metabolic process	GO:0009132		0.036510	**0.000640**	**0.000564**	**0.000882**	**0.000510**	0.03651	**0.00056**
system process	GO:0003008		**0.000640**	0.036510	**0.000564**			**0.00241**	**0.00212**
carbohydrate catabolic process	GO:0016052			**0.002413**	**0.002126**			0.02205	0.01786
carboxylic acid metabolic process	GO:0019752			0.022050	0.017865			0.02063	0.01823
monocarboxylic acid metabolic process	GO:0032787			0.020630	0.018232			0.02383	0.01929
oxoacid metabolic process	GO:0043436			0.023804	0.019293			0.02664	0.02160
organic acid metabolic process	GO:0006082			0.026642	0.021605			0.03437	0.02913
generation of precursor metabolites and energy	GO:0006091			0.034376	0.029131			0.03437	0.029131
striated muscle thin filament	GO:0005865	**0.000240**	**0.000001**	**0.000168**	**0.000000**	**0.000001**	**0.000000**	**0.000207**	**0,000000**
myofilament	GO:0036379	**0.000240**	**0.000001**	**0.000168**	**0.000000**	**0.000001**	**0.000000**	**0.000207**	**0,000000**
sarcomere	GO:0030017	**0.001119**	**0.000005**	**0.000785**	**0.000004**	**0.000008**	**0.000003**	**0.000969**	**0.000003**
myofibril	GO:0030016	**0.001488**	**0.000008**	**0.001044**	**0.000006**	**0.000012**	**0.000004**	**0.001289**	**0.000005**
contractile fiber	GO:0043292	**0.001929**	**0.000011**	**0.001353**	**0.000008**	**0.000018**	**0.000006**	**0.001671**	**0.000008**
supramolecular polymer	GO:0099081	**0.003933**	**0.000098**	**0.002252**	**0.000067**	**0.000194**	**0.000042**	**0.003089**	**0.000066**
supramolecular fiber	GO:0099512	**0.003933**	**0.000098**	**0.002252**	**0.000067**	**0.000194**	**0.000042**	**0.003089**	**0.000066**
muscle thin filament tropomyosin	GO:0005862	0.010816	**0.008413**	**0.008413**	**0.007279**	0.010816	**0.005832**	**0.009856**	**0.007278**
cytoskeleton	GO:0005856	0.017549	**0.000801**	**0.008339**	**0.000486**	**0.001941**	**0.000265**	0.012609	**0.000485**
supramolecular complex	GO:0099080	0.021834	**0.000806**	0.012671	**0.000554**	**0.001572**	**0.000347**	0.017266	**0.000553**
polymeric cytoskeletal fiber	GO:0099513	0.042641	0.027374	0.027374	0.021312	0.042641	0.015254	0.035404	0.021311
actin cytoskeleton	GO:0015629				**0.003417**	**0.006938**	**0.002434**		**0.003417**
actin filament	GO:0005884						0.043209		
troponin complex	GO:0005861				**0.003647**	**0.005422**	**0.002922**		0.003647
Glycolysis / Gluconeogenesis	KEGG:00010	**0.002318**	**0.000854**	**0.000059**	**0.000024**	**0.002318**	**0.000854**	**0.000058**	**0.000023**
Carbon metabolism	KEGG:01200	0.041924	0.016114	**0.002453**	**0.001026**	**0.041924**	**0.016114**	**0.002453**	**0.001025**
Biosynthesis of amino acids	KEGG:01230		0.043307	**0.004533**	**0.002223**		**0.043307**	**0.004533**	**0.002223**

In bold p value ≤ 0.005.

Regarding resistant strain parasites, it is notable the finding of eight proteins exclusively present in those *S. mansoni* adult parasites. From those eight proteins, dopamine transporter, twister (putative), ubiquitin-specific peptidase 30 (C19 family), and uncharacterized protein smp_162220 were not detected in the proportion of the proteome we analysed in *S. mansoni* PZQ-exposed adult parasites. However, galactokinase, ornithine aminotransferase, Rab6-interacting protein 2 (ERC protein 1),

and transducin beta-like remained after drug exposure. Dopamine/norepinephrine transporter (*SmDAT*) gene transcript, characterized in *S. mansoni*, is essential for the survival of the parasite as it causes muscular relaxation and a lengthening in the parasite, controlling movement (86). Galactokinase catalyzes the second step of the Leloir pathway, a metabolic pathway found in most organisms for the catabolism of β-D-galactose to glucose 1-phosphate (87). Galactokinase and hexokinase have a similar enzymatic function on sugar phosphorylation (88), and characterization of schistosome hexokinase has been described as pertinent to understanding the metabolic response of *S. mansoni* cercariae to an increased glucose availability (89). Ornithine aminotransferase was already identified in *S. mansoni* (90) and it has been characterized as playing a central role in ornithine biosynthesis (91). It seems responsible for catalyzing the transfer of the delta-amino group of L-ornithine to 2-oxoglutarate, producing L-glutamate-gamma-semialdehyde, which in turn spontaneously cyclizes to pyrroline-5-carboxylate, and L-glutamate (92). Rab6-interacting protein 2 is a member of a family of RIM-binding proteins, which are presynaptic active zone proteins that regulate neurotransmitter release (93). Ubiquitin-specific peptidase 30 (C19 family) belongs to a metabolic pathway that had previously been associated with the development of artemisinin and artesunate-resistance in *Plasmodium chabaudi* (94), which is a very interesting result. All those proteins specifically found in the resistant strain should be further studied to better understand if they could have a fundamental role in PZQ-resistance development.

Yet, for the resistant strain parasites, there are three proteins, beta 1,3-galactosyl-transferase, cathepsin L, and receptor for activated Protein Kinase C (PKC) that are exclusive to resistant females, even after exposure to PZQ. Beta 1,3-galactosyltransferase has previously been identified in the *Schistosoma* genome and transcriptome (77). Cathepsin L activity is believed to be involved in hemoglobin digestion by adult schistosomes (95), and these authors suggested the involvement of cathepsin proteinases in several key functions render them as potential targets to novel antiparasitic chemotherapy and immunoprophylaxis. Putative PKC exists in kinomes of the blood flukes *S. mansoni* (20, 96, 97), *S. japonicum* (98), *S. haematobium* (99), and regulates movement, attachment, pairing, and egg release in *S. mansoni*, being considered a potential target for chemotherapeutic treatment against schistosomiasis (100). These results need further investigation thus considering that the PZQ mode of action is not completely clarified nor is its genetic process of susceptibility lost, our results might help in the process of clarification of a possible relationship between those proteins and PZQ-resistance in *S. mansoni* females (20, 45). Concerning the PZQ-susceptible strain, we noted eight proteins that only appeared in this strain. Phosphopyruvate hydratase (enolase), an important glycolytic enzyme that has the functions of activating the plasminogen, involving in the processes of infection and migration of parasites, reducing the immune function of the host as well as preventing parasites from the immune attack of the host (101), is the only protein from those eight proteins that continued to be present after PZQ exposure.

Our gene enrichment analyses also provided functional differences when comparing males and females. The results identified in susceptible females, either exposed or not exposed, a significantly higher p-value for GO terms that were related to ATP generation from ADP and other GO terms within the ancestor chart of this term. On the other hand, susceptible males presented significantly higher p-values for GO terms associated with muscle contraction. This give us an indication of a higher fitness and active metabolic status of susceptible females both in the presence or absence of Praziquantel when compared to susceptible males. Also, processes related to monocarboxylic acid metabolic processes were identified only in susceptible males and females, both exposed and unexposed. This could also suggest that loss of susceptibility/resistance to Prazinquantel come at the compromise of the fitness level, with lower metabolic levels both in males and females.

These results altogether represent an important finding for the study of PZQ-resistance/susceptibility in *S. mansoni*, since they allow comparing directly the proteome under both conditions. We believe that the most promising candidates are proteins that appeared associated only to one of the strains, especially those with functions possibly related to the phenotypic alterations observed by Pinto-Almeida et al. (11), or previously associated with resistance by other parasites to different drugs. These candidates require special attention in more studies, assessing for instance the level of protection induced by these proteins in animal models infected by both *S. mansoni* strains, as they may have some involvement in the PZQ-resistance phenomenon, although further studies are needed to deepen this knowledge.

CONCLUSIONS

Here, we investigated the proteome of a PZQ-resistant *S. mansoni* strain and the respective isogenic susceptible strain, an important step towards a full clarification on the genetic process of PZQ-resistance considering that we were able to compare a PZQ-resistant parasite line with the susceptible one from with it was obtained allowing a more accurate comparison between strains that differ solely on PZQ susceptibility phenotype. This study allowed us to identify proteins that in our proteome analyses differ between PZQ-susceptible and PZQ-resistant parasites, however, their relationship or contribution towards the mechanism of resistance remains unclear and strongly needs further clarification. Since these proteins may be involved with the PZQ-resistance phenomenon, their functional characterization should be pursued in future studies aiming at the discovery of new drug targets for schistosomiasis control and also studies that could clarify the PZQ mode of action.

The identification of the proteins putatively associated with PZQ-resistance in *S. mansoni* permits also to investigate the possibility of developing a diagnostic test to distinguish patients carrying PZQ-resistant strains from those with PZQ-susceptible *S. mansoni*. The development of such a test would constitute a major step towards schistosomiasis control as it would render it

possible to adjust drug administration to increase treatment efficacy, perhaps even by combining PZQ with efflux pump inhibitors as suggested previously (45). The proteome analysis made it also possible to identify proteins that were present only in females, being them good targets to identify the mechanisms underlying the decreased PZQ-susceptibility of females, when compared to males. Furthermore, some of these proteins may constitute targets, for schistosomiasis control. They should, therefore, be the object of further analysis. In this context, the development and use of other techniques, such as genetic manipulation methods, will be crucial to further unravel the phenomenon/problem of PZQ-resistance/tolerance/loss of susceptibility. Therefore, this work opens doors to other PZQ-resistance studies, and could represent a basis to find a solution to the PZQ-resistance problem in a disease that affects millions of people worldwide.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://datadryad.org/stash/share/_nzZNotPFuNIsBY1R5x1XjDy3XUFKGAEsGBDFNl3yWM.

ETHICS STATEMENT

The research project was reviewed and approved by the Ethics Committee and Animal Welfare, Faculty of Veterinary Medicine, UTL (Ref. 0421/2013). Animals were maintained and handled in accordance with National and European legislation (DL 276/2001 and DL 314/2003; 2010/63/EU adopted on 22nd September 2010), with regard to the protection and animal welfare, and all procedures were performed according to National and European Legislation. The anesthetics and other techniques were used to reduce the pain and adverse effect of animal.

AUTHOR CONTRIBUTIONS

AP-A: Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing - original draft, writing - review and editing, and visualization. TM: Conceptualization, methodology, investigation, resources, writing - original draft, and writing - review and editing. PF: Writing - original draft, writing - review and editing, and experimental studies. ABA: Writing - review and editing, and gene ontology analysis. SB: Conceptualization, writing - original draft, and writing - review and editing. FA: Writing - original draft, writing - review and editing, and funding acquisition. SA: Resources, writing - original draft, and writing - review and editing. CG: Writing - review and editing. EC: Conceptualization, methodology, software, validation, formal analysis, data curation, writing - original draft, writing - review and editing, visualization, supervision, project administration, and funding acquisition. AA: Conceptualization, methodology, software, validation, formal analysis, investigation, research, data curation, writing - original draft, writing - review and editing, visualization, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2021.664642/full#supplementary-material and https://datadryad.org/stash/share/_nzZNotPFuNIsBY1R5x1XjDy3XUFKGAEsGBDFNl3yWM

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