The Characterization and Proteomic Analysis of *Schistosoma mansoni* Cercariae Extracellular Vesicles

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Abstract

Schistosomiasis is a neglected tropical disease and a major public health problem caused by *Schistosoma* species. Chronic schistosomiasis is characterized by egg granulomas found in the liver or bladder, depending on the causative *Schistosoma* species of infection. This water-borne parasite currently has one widely used treatment, praziquantel (PZQ), which has shown reduced efficacy against all *Schistosoma* species. However, PZQ is only effective against the adult worms and it is the egg-stage which drives disease pathology. Additionally, it does not protect against reinfection. Exosomes are extracellular vesicles (EVs) that are multi-vesicular bodies involved in intracellular communication. Since exosomes contain pathogen-associated molecules which have been shown to induce modifications in non-infected neighboring cells or act as antigens for immune cells, the study of *S. mansoni*-derived exosomes is of interest. Many groups have investigated the contents and the roles of *Schistosoma* EVs, particularly those derived from adult worms and eggs. However, none have studied the contents and possible roles of EVs derived from *S. mansoni* cercariae. Since the cercaria life cycle stage is actively infecting the host through the skin, EVs secreted at the time of penetration may aid in understanding the host-parasite relationship further. Therefore, we hypothesize that through the proteomic analysis of EVs secreted by *S. mansoni* cercariae during infection may aid in understanding the host-parasite interface at the site of infection. With liquid chromatography tandem mass spectrometry, we were able to identify 25 proteins derived from EVs secreted during cercariae-schistosomula transformation. The identification of proteins was accepted if it had a 95% minimum probability and at least two identified peptides. Some of which are known exosome markers such as heat shock protein 70, tetraspanins like CD63, and enolase. We also identified a known vaccine candidate Sm29 in the EVs. Additionally, other proteins found are shown to be involved in cellular processes and signaling such as protein synthesis and calcium mediated signal transduction further supporting our hypothesis. Therefore, not only is the study of EVs secreted by *S. mansoni* cercariae vital to the understanding of the host-parasite relationship but it also may lead to vaccine or treatment development.

Résumé

La schistosomiase est une maladie tropicale négligée et un problème majeur de santé publique causé par les espèces de *Schistosoma*. La schistosomiase chronique est caractérisée par des granulomes d'œufs trouvés dans le foie ou la vessie selon l'espèce de *Schistosoma* responsable de l'infection. Cette maladie peut en fait être traitée par un médicament largement utilisé, le praziquantel (PZQ), qui a fait preuve d'une bonne efficacité contre toutes les espèces de *Schistosoma*. Cependant, le PZQ n'est efficace que contre les vers adultes et c'est le stade de l'œuf qui est responsable des symptômes plus sévères de la maladie. De plus, il ne protège pas contre la réinfection. Les exosomes sont des vésicules extracellulaires (VEs) qui sont des corps multivésiculaires impliqués dans la communication intracellulaire. Étant donné que les exosomes contiennent des molécules associées à des agents pathogènes qui induisent des modifications dans les cellules voisines non infectées ou agissent comme des antigènes pour les cellules immunitaires, l'étude des exosomes dérivés de *S. mansoni* est intéressante. De nombreux groupes ont étudié le contenu et les rôles des VEs de *Schistosoma*, en particulier ceux dérivés de vers adultes et d'œufs. Cependant, aucun n'a étudié le contenu et les rôles possibles des VEs dérivés de larves cercaires de *S. mansoni*. Étant donné que le stade du cycle de vie des cercaires infecte activement l'hôte à travers la peau, les VEs sécrétés au moment de la pénétration peuvent aider à mieux comprendre la relation hôte-parasite. Par conséquent, nous émettons l'hypothèse que l'analyse protéomique des VEs sécrétés par S. mansoni cercariae pendant l'infection peut aider à comprendre l'interface hôte-parasite sur le site de l'infection. Avec chromatographie liquide spectrométrie de masse en tandem, nous avons pu identifier 25 protéines dérivées des VEs sécrétées lors de la transformation cercaires-schistosomules. L'identification des protéines était acceptée si elle avait une probabilité minimale de 95 % et au moins deux peptides identifiés. Certains d'entre eux sont des marqueurs d'exosomes connus tels que la protéine de choc thermique 70, les tétraspanines comme CD63 et l'énolase. Nous avons également identifié un candidat vaccin Sm29 connu dans les VEs. De plus, d'autres protéines trouvées sont impliquées dans les processus cellulaires et la signalisation telle que la synthèse des protéines et la transduction du signal médiée par le calcium soutient davantage notre hypothèse. Par conséquent, non seulement l'étude des VEs sécrétés par les cercaires de *S. mansoni* est vitale pour la compréhension de la relation hôte-parasite, mais elle peut également conduire au développement d'un vaccin ou d'un traitement.

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Preface

This Master's thesis was written in accordance with McGill's University's "Guidelines for Thesis Preparation". The candidate has chosen to present her thesis as a "manograph-based thesis" or traditional style thesis. All work towards this thesis was performed under the co-supervision of Dr. Momar Ndao (primary supervisor) and Dr. Martin Olivier (secondary supervisor). The candidate is the first author of the thesis.

Author contributions are as followed:

Overall project was designed and envisioned by Momar Ndao (MN) and Martin Olivier (MO). *In vitro* experiments were designed and performed by Selena Yu (SY), MN, and MO. Transmission electron microscopy imaging was performed by SY with aid from George Dong (GD). Nanoparticle tracking analysis measurements was performed by Laura Montermini from the lab of Janusz Rak with help from GD. Preparation of samples for LC-MS/MS and proteomic analysis was performed by SY with help from GD. Denis Faubert from the Recherches Cliniques de Montréal (IRCM, University of Montréal) preformed LC-MS/MS on the samples. Manuscript was written by SY with guidance from MN and MO. Manuscript proofreads and edits made by Adam Hassan, Dilhan Perera, MN, and MO. French abstract written by AH.

List of Abbreviations

aaMΦ	alternative activated macrophages
BCE	before common era
CAA	circulating anodic antigen
CCA	circulating cathodic antigen
CD	cercarial dermatitis
CE	common era
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
ENI	endemic normal individual
ESCRT	endosomal sorting complexes required for transport
EV	extracellular vesicle
HIV	human immunodeficiency virus
IFN	interferon
IHA	indirect hemagglutination
IL	interleukin
ILV	intraluminal vesicle
KS	katayama syndrome
LC-MS/MS	liquid chromatography tandem mass spectrometry
MDA	mass drug administration
MVB	multivesicular body
MΦ	macrophage
NTA	nanoparticle tracking analysis
NTD	neglected tropical disease
PCR	polymerase chain reaction
PZQ	praziquantel
RAG	recombination activating gene
SEA	soluble egg antigens
Sh28GST	*S. haematobium* Recombinant glutathione S-transferase
SmCB	*S. mansoni* Cathepsin B
TEM	transmission electron microscopy

TGF	tumour growth factor
TNF	tumour necrosis factor
WASH	water, sanitation, and hygiene
WHO	World Health Organization

Chapter 1: A literature review of schistosomiasis and exosomes

1.1 Epidemiology of Schistosomiasis

The World Health Organization estimates that over 200,000 deaths per year are attributed to schistosomiasis and over 700 million people are at risk for infection [1]. The genus *Schistosoma* are trematode worms that cause schistosomiasis which is a fresh-water-borne disease. This makes schistosomiasis a high morbidity and mortality neglected tropical disease (NTD). However, since current diagnostic tools may not detect low-intensity infections and access to remote endemic villages is limited, this disease burden is believed to be underestimated [2]. According to the Global Burden of Disease Study 2016, the burden of schistosomiasis is 1.9 million disability-adjusted life years (DALYs) [2]. The DALYs rate is influenced by many schistosomiasis-associated conditions and its effect on growth and cognitive development in children [2]. Unfortunately, DALYs do not include the economic and social impact of NTDs such as school attendance, agriculture, and overall economic productivity [2]. However, the DALYs has decreased since it was recorded in the 2010 project as 3.3 million [3]. This may be due to increased disease surveillance, mass administration of praziquantel, and snail control [2].

Schistosomiasis, and other NTDs, cases are concentrated in tropical and sub-tropical regions, particularly in areas where water, sanitation, and hygiene (WASH) conditions are poor [2-4]. Additionally, many communities with high cases of schistosomiasis participate in water activities such as fishing, domestic activities and swimming in contaminated water [2]. Therefore, improvement in WASH conditions and mass drug administration (MDA) is necessary for controlling and eliminating NTDs [2-4]. There are six *Schistosoma* species that infect humans, *S.guineensis, S.haematobium, S.intercalatum, S. japonicum, S. mansoni,* and *S.mekongi* [5]. The geographical distribution of different *Schistosoma* species varies. *S. intercalatum* and *S. guineensis* are found in West and Central African regions [6]. *S. haematobium* is found in Africa, India, and the Middle East [5, 7]. *S. japonicum* is endemic in the People's Republic of China, Philippines, Indonesia, and Japan [7, 8]. *S. japonicum* can also infect cattle, water buffalo, horses, cats, dogs, and other animals [9]. It is estimated that 90% of *S. japonicum* transmission in Chinese farm districts is attributed to water buffalo [8]. *S. mansoni* is found in Africa, the West Indies, and South America (especially in Brazil, Venezuela, and the Caribbean Islands) [2, 3, 7]. Lastly, *S. mekongi* is found only in the Mekong river basin of Cambodia [10]. Due to an increase in travel and immigration, schistosomiasis has been reported in Europe and North America. Between 1997 to

2010, the European Network for Tropical Medicine and Travel Health reported 1,465 cases of imported schistosomiasis [11]. Therefore, it is vital to inform travelers of the risk of schistosomiasis and screen travelers post-travel to detect infections [11].

There are numerous methods in which the global burden of schistosomiasis can be decreased and eventually eliminated. The epidemiology of schistosomiasis has changed over the years due to different disease control methods such as snail control, irrigation, mass administration of drugs, and poverty reduction [7, 12]. WHO announced in March 2020 that there needs to be a refocus on snail control to sustain schistosomiasis elimination [13]. Snail control and/or changes in their habitat have been shown to interrupt transmission in endemic regions [7, 14]. In 2020, WHO published a manual on the use of molluscicides in the field [15]. The steps to successful snail control include planning the snail control program, snail sampling and application of molluscicides, and monitoring and evaluating progress [15]. Niclosamide-derived molluscicides are highly active against all stages of the snail life cycle and schistosome larvae and are safe in the presence of livestock and poultry [15]. Unfortunately, niclosamide is toxic to fish and amphibia [15]. Therefore, it has a negative impact of the environment and biodiversity [7]. Niclosamide also does not prevent snails from recolonizing their original habitat [7]. However, with a proper plan and administration, niclosamide can be used to control schistosomiasis. Another possible molluscicide is linalool which is a *Cinnamomum camphora* extract [16]. Linalool kills *Oncomelania hupensis* snails, the intermediate host of *S. japonicum*, and can be used to treat *S. japonicum* infections in infected BALB/c mice [16]. Snails treated with linalool showed gill destruction, cell degradation, and shrunk hepatopancreases, leading to their deaths. Importantly, linalool extracts have not been shown to have environmental risks [7]. Lastly, using dried leaves of *Ambrosia maritima*, a plant molluscicide, in schistosome infected creeks have been shown to decrease *Bulinus* species by 54-56% after two weeks of treatment [17]. When used in closed irrigation water systems, *A. maritima* reduced the density of *Biomphalaria pfeifferi* by 77% in two weeks [17].

The creation of dam reservoirs and irrigation systems lead to habitat expansion of intermediate host snails and thus create new transmission sites for schistosomiasis [12]. It is estimated that 106 million people who are at risk for schistosomiasis, out of over 700 million people, live in irrigation schemes or close proximity to large dam reservoirs [12]. For example, in Senegal, there was an increase in irrigated areas between 1997 and 1999 which corresponded to

an increase in *S. haematobium* prevalence, from 27% to 48% [12]. Additionally, in Northern Senegal, the contraction of the Diama barrage close to the estuary of the Senegal river in 1985, blocked the intrusion of saltwater into the river which allowed for large-scale irrigation [12, 18]. *S. mansoni* infections were low in children and young adults prior to the closure of the Diama dam [12, 18]. However, 18 months after the closure, the prevalence of *S. mansoni* increased from 4.4% to 43.5% in endemic villages in the delta [12, 18].

Currently, the best and most used drug for treatment and morbidity control of schistosomiasis in sub-Saharan Africa is praziquantel (PZQ) [19]. PZQ kills the adult worms in the host through an unknown mechanism [1]. However, the early effects of the drug are known, and they are: (1) calcium influx into the whole parasite, (2) muscle contractions, and (3) surface tegument modifications [20]. MDA programs aim to administer treatment, 40 mg/kg, to every individual in communities independent of their disease status [7]. MDA of PZQ has significantly reduced the global burden of disease and reduced egg excretion in some low transmission areas such as the Nile Delta and Brazil [7]. However, to have successful disease elimination, other measures such as snail control, increased diagnosis, and education on WASH, need to be implemented [7]. This is because PZQ does not prevent reinfection, therefore control programs need to focus not only on morbidity control, but also on sustainable control of schistosomiasis [7]. Controlling the prevalence of the intermediate host in high-risk communities have been shown to interrupt transmission [7, 19]. The use of molluscicides to control snail populations have been shown to enhance the impact and performance of MDA campaigns and case management [21]. For example, in Venezuela, the prevalence of *S. mansoni* infections in an endemic area was 14% in 1943 [22]. However, by 1996, the prevalence dropped to 1.4% due to the elimination of the snail intermediate host, reducing human contact with contaminated water, health education, and drug administration [22]. Access to clean water, proper sanitation, and good hygiene are essential in combating schistosomiasis as it breaks the cycle of transmission [21]. Since schistosomiasis heavily affects poorer populations, it is crucial to improve the socioeconomic standing of endemic regions to eliminate the disease.

1.2 The Lifecycle of Schistosoma

The most common *Schistosoma* species to infect humans are *S. mansoni*, *S. japonicum,* and *S. haematobium*. Feces or urine from *Schistosoma* infected hosts contain parasite eggs. In the case

of *S. mansoni,* the eggs (142 μm in length x 60 μm in width) hatch and release miracidia (140 μm in length x 66 in width μm) [23] when in fresh water. Each *Schistosoma* species requires a specific snail intermediate host to continue its life cycle. In the case of *S. mansoni,* the snail host is *Biomphalaria* species whereas *S. japonicum* infects *Oncomelania* genus, and *S. haematobium* miracidia infects *Bulinus* species [23]. In hepatopancreas, there will be two mother-to-daughter generations of sporocysts followed by daughter to sporocysts which differentiate into cercariae [24]. The length of the emerging cercariae depends on the snails' ambient temperature and light (300-500 μm) [23]. The cercaria larval stage infects their human host by penetrating the host skin [25]. It has been shown that lipids on the surface of the skin are one of the triggers of schistosome invasion [26-29]. During invasion, the cercariae tails and two sets of acetabular glands are released [26-29]. The acetabular glands contain proteolytic enzymes, several proteins involved in immune evasion, and adhesion mucin-like substance [26-29]. The contents of the acetabular glands facilitate penetration and immunomodulation in the host epidermis [26-29]. Furthermore, the transforming cercariae will shed their thick tegumental glycocalyx, an immunogenic structure, to help the parasite avoid immune detection [30, 31]. Additionally, the cercaria will shed its forked tail upon entry and tegument membranes once inside the host, allowing for its transformation into the migrating schistosomula [29]. *Schistosoma* invasion through the epidermis is facilitated by acantholysis which degrades the epidermal basement membrane, allowing schistosomula access to the small venules or lymphatics [26, 32]. It has been demonstrated that *S. mansoni* schistosomula can remain in the skin for up to six days [33]. However, the mean rate of migration of the schistosomula in the dermis ranges from 55 to 79 hours [34]. The schistosomula will enter the venules in the skin and travel to the pulmonary vasculature [35]. The migrating larvae possess the ability to uptake nutrients and will undergo morphological changes [35]. The lung resident schistosomula will increase up to four-fold in length, to accommodate passage along the lumen of pulmonary and systemic capillaries [36, 37]. Additionally, they will lose their mid-body spines to facilitate transit [38]. However, as shown in a mouse model, a proportion of *S. mansoni* schistosomula becomes trapped and diverted to the air spaces which can be coughed up, swallowed, and digested [39]. The larvae begin to leave the lungs five to seven days post-infection via the venous circulation to the left side of the heart [38]. Approximately one-third of schistosomula enter the portal system by passing through the gastrointestinal capillary beds and the splanchnic arteries [38]. The remaining schistosomula population re-enters the lungs through

the capillary beds of systemic organs [38]. Due to schistosomula migration through the host's circulation, some parasites may get trapped in the brain, heart, and diaphragm [36]. The larvae will reach the hepatic portal system between 7-21 days and then will mature for a three weeks before *S. mansoni* and *S. japonicum* adult worms form mating pairs [40]. *S. japonicum* larvae will migrate to the small venules in the submucosa of the bladder wall and then to the perivesical venous plexus [41]. After maturing, the adult *S. japonicum* worms will then form mating pairs[41]. The *in copula* pairs will migrate to the veins of the intestine walls [40]. The slender female (7.2 – 14 mm in length) is fitted into the male (6 – 13 mm in length) gynecophoric canal, in the mesenteric venules [23, 25]. *S. mansoni* adult worms reside in the mesenteric veins draining the large intestines. *S. japonicum* adults are found in the mesenteric veins draining the small intestines. *S. haematobium* adults reside in the veins surround that surround bladder [25]. The adult *S. mansoni* females can lay >300 eggs/day whereas *S. japonicum* females lay >2000 eggs/day and *S. haematobium* females lay >200 eggs/day [42, 43]. Adult worms can live 3-10 years in their hosts [44]. *S. mansoni* and *S. japonicum* eggs are excreted via feces, and *S. haematobium* eggs are excreted in the urine [25]. Eggs released into fresh water with the schistosome species specific snail host will restart the life cycle.

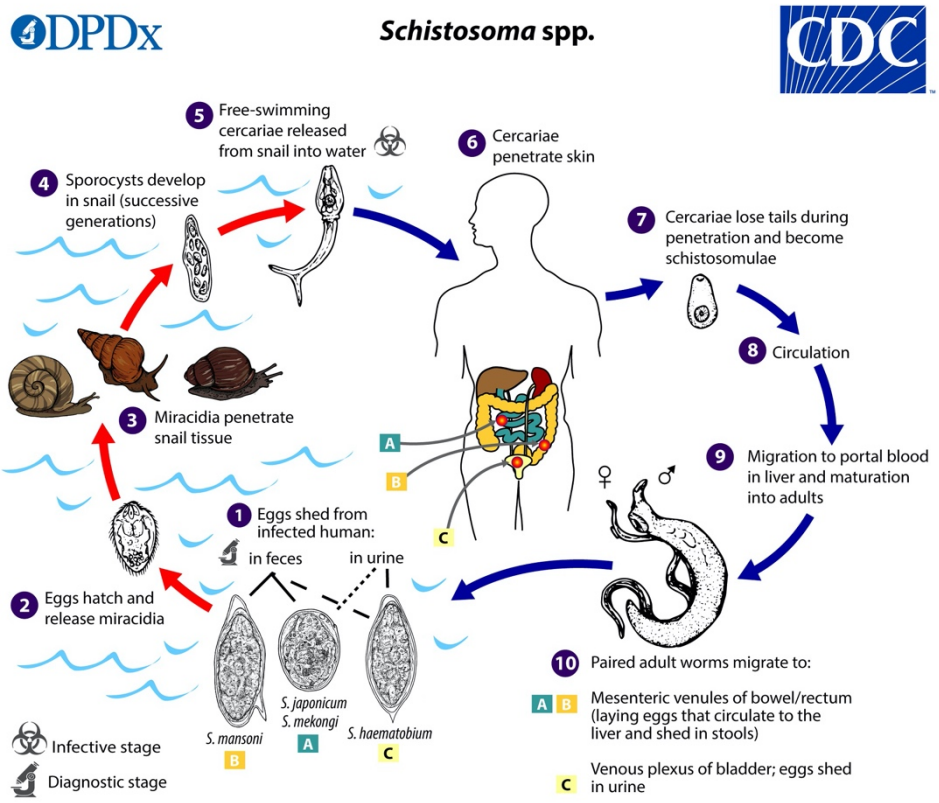

Figure 1: Life cycle of Schistosoma spp.

Schistosoma miracidia will hatch from eggs in fresh water. The miracidia will penetrate the intermediate snail host where the parasite will mature. The cercariae will leave the snail and penetrate the skin of the human host. The cercariae will transform into schistosomula and travel through the circulation. The schistosomula will mature into female and male adult worms. The adult worms will pair off *in copula*. The females produce eggs which are released into the environment via the urine in the case of *S. haematobium* or via the feces for *S. japonicum* and *S. mansoni*.

Figure derived from Centers of Disease Control and Prevention. 2019 (2019). Biology. Schistosomiasis. Retrieved from https://www.cdc.gov/parasites/schistosomiasis/biology.html

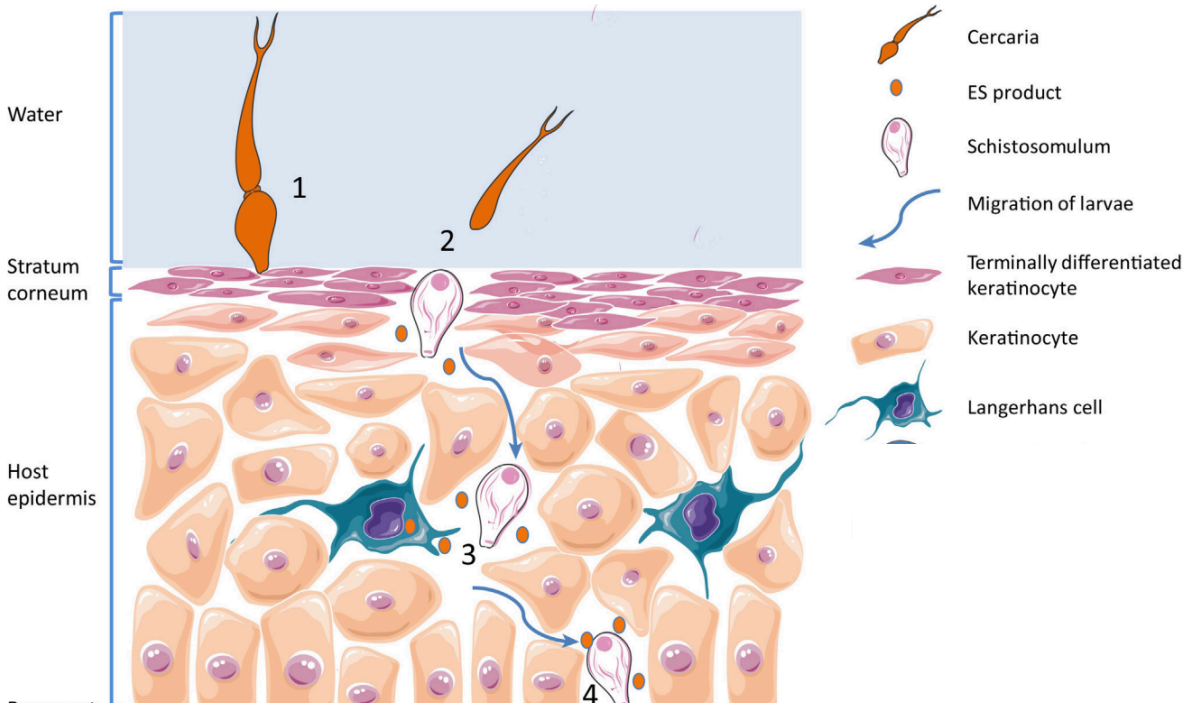

Figure 2: Migration of schistosomula through the human host skin

Schistosoma cercariae penetrate human skin and lose their forked tails. Additionally, excretory/secretory (E/S) products that contain proteolytic enzymes and extracellular vesicles, for example, are released into the host. These E/S products will interact with host immune cells like resident Langerhans cells to initiate the innate immune response.
Figure derived from Egesa, M., Hoffmann, K. F., Hokke, C. H., Yazdanbakhsh, M., & Cose, S. (2017). Rethinking schistosomiasis vaccine development: synthetic vesicles. *Trends in parasitology, 33*(12), 918-921.
Modified by SY.

1.3 Clinical Manifestations of Schistosomiasis

1.3.1 Acute schistosomiasis

Acute schistosomiasis is characterized by cercarial dermatitis (CD) and Katayama Syndrome (KS) [45-47]. CD is a hypersensitivity response against penetrating cercariae [45-47]. Interestingly, CD is only commonly seen in tourists and foreigners visiting schistosomiasis endemic regions and after primary infections [6, 48, 49]. In particular, CD is characterized by a

maculopapular, pruritic rash that occurs within a couple hours of exposure to contaminated waters and can persist for several days [47]. On the other hand, KS is an immune-complex mediated hypersensitivity reaction against the migrating schistosomula and early egg deposition [45-47]. KS symptoms begin to appear 14-84 days post schistosome exposure [45-47]. KS occurs almost exclusively in nonimmune travelers to schistosomiasis endemic regions [45-47]. Some symptoms of KS are rapid onset fever, fatigue, headache, malaise, myalgia, non-productive cough, and eosinophilia with patch infiltrates that are visible on pulmonary radiography [45-47, 50]. Some individuals may also experience abdominal symptoms caused by the migration of juvenile [46, 47, 51]. Acute schistosomiasis caused by *S. mansoni* and *S. haematobium* is common in individuals' experiencing their first exposure such as travelers and migrants but rare among endemic populations [6, 47, 50]. On the other hand, acute schistosomiasis caused by *S. japonicum* is common in individuals that live in endemic region and they experience severe manifestations that progress to hepatosplenomegaly and portal hypertension [48].

1.3.2 Chronic schistosomiasis (Genitourinary and Gastrointestinal)

Chronic schistosomiasis has different manifestations depending on the *Schistosoma* species as their adult worms vary in anatomical location within the vasculature of the mammalian host [45]. *S. haematobium* chronic infections cause genitourinary disease [45-47]. Whereas *S. mansoni* and *S. japonicum* adults infect the hepatointestinal and hepatosplenic regions [45-47]. Furthermore, the severity of chronic schistosomiasis can be influenced by co-infections such as by hepatitis B and C viruses, human immunodeficiency virus (HIV), and malaria [7, 47]. Severe schistosomiasis is due to poor immunoregulation of anti-schistosome egg responses, not the adult worms themselves [49].

S. haematobium is the main cause of chronic genitourinary schistosomiasis [6, 47]. The adult *S. haematobium* worms live in the vasculature surrounding the genitourinary tract [6, 47]. The eggs laid by the females are embedded in the bladder and ureter walls. The chronic *S. haematobium* infection causes fibrosis and calcification of the bladder and ureters which allows for an opportunistic secondary bacterial super-infection [6, 47, 50]. Chronic genitourinary schistosomiasis manifests as renal colic, hydroureter, hydronephrosis, and renal failure [6, 47, 50]. Importantly, genitourinary schistosomiasis induced bladder cancer has been reported in Egypt and other regions in Africa [6, 47, 50]. Male genital schistosomiasis is characterized by

haematospermia, orchitis, prostatitis, dyspareunia, and oligospermia [6, 50, 52, 53]. Also, genital schistosomiasis conditions in men can be resolved more readily with anti-schistosomal treatment than women [52, 53]. Genital schistosomiasis develops in a third of *S. haematobium* infected women which can manifest as female genital schistosomiasis [50]. Egg granulomas are formed in the vulva, vagina, or cervix, and produce ulcerative lesions [6, 50]. Not only do these inflammation lesions increase the risk for the transmission of sexually transmitted infections, but the fibrotic scarring may lead to infertility and increased risk of abortion [6, 50]. A study in Zambia demonstrated that *S. haematobium* infections are associated with increased HIV transmission in both sexes [54]. Additionally, HIV+ women infected with *S. haematobium* have increased death progression [54]. HIV target cells in *S. mansoni* infected female genital mucosa which allows for increased HIV transmission [55].

Gastrointestinal schistosomiasis pathology is caused by a granulomatous response to schistosome eggs embedded in the intestinal mucosa [7, 50]. The egg granulomas lead to pseudeopolyposis, microulceration, and superficial bleeding [7, 50]. Other symptoms include chronic or intermittent abdominal pain and discomfort, diarrhea, and loss of appetite [7, 50]. Hepatosplenic schistosomiasis is caused by the formation of granulomas around egg deposits in the hepatic sinusoids [7, 50]. Chronic intestinal schistosomiasis leads to the excess deposition of extracellular matrix (ECM) components, like collagen, in the liver [7, 50]. The accumulation of ECM components leads to periportal fibrosis and progressive occlusion of the portal veins [7, 50]. The occlusion of the portal veins is followed by portal hypertension, portacaval shunting, ascites, gastrointestinal varices and bleeding, and splenomegaly [47, 51]. *S. mansoni* infections progress to chronic hepatosplenic schistosomiasis within 5-15 years, whereas *S. japonicum* chronic disease progresses more rapidly [6].

1.4 Immunopathology of chronic schistosomiasis

The host's immune response to parasite eggs in tissues causes the disease manifestations of schistosomiasis. The hepatoxic soluble egg antigens (SEAs) are a persistent source of antigen stimulus and elicit a granulomatous response [56-59]. Studies with mice, rhesus monkeys, and pigs have shown that the granulomatous response around the eggs develop through five pathological stages: weakly reactive, exudative, exudative-productive, productive, and involutional stages [56-59]. Burke *et al*., demonstrated that SEA induces surrounding macrophages

to secrete inflammatory cytokines and chemokines that recruit lymphocytes, neutrophils, and monocytes needed for granulomatous inflammation [60]. Excessive tissue damage which can lead to premature death, is limited by these granuloma components [61]. Moreover, a clinical study has shown that chronically infected patients have decreased levels of IFN-γ and TNF-α in their plasma compared to acutely infected patients [62]. Therefore, the presence of IFN-γ and other pro-inflammatory cytokines is an indication of the acute granulomatous response [62]. Additionally, the T-cell response plays a vital role in developing the granulomatous response and host survival. When T-cell deficient mice were infected with *S. mansoni*, they suffered from severe necrosis around hepatic eggs and died prematurely [63].

There is a switch from a T-helper type 1 to T-helper type 2 (Th1 to Th2) during infection. In the first 4-6 weeks of infection, Th1 response is elicited against the migrating schistosomula and immature adult worms [45, 64-67]. The Th1 response is characterized by increased levels of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IFN-γ [45, 64-67]. The immune switch from Th1 to Th2 occurs at the onset of egg-laying. The Th2 response is characterized by the expression of IL-4, IL-5, IL-10, and IL-13 cytokines which is down-modulated with disease progression [45, 64-68]. The essential pathway that generates the switch from Th1 to Th2 is the IL-4/IL-13 mediated signal transducer and activator of transcription 6 (STAT6) pathway [69]. Kaplan *et al.* demonstrated that lymphocytes from *S. mansoni* infected STAT6-deficient mice produce minimal levels of Th2 cytokines, have enhanced IFN-γ production, and smaller granulomas[70]. IL-4 is the key driver of the granulomatous response and IL-13 is the cytokine responsible for the development of hepatic fibrosis [69]. IL-4 is responsible for granuloma size and inducing the proliferation of Th2 cytokine-producing lymphocytes [45, 69, 71-75]. Furthermore, IL-4 induces the production of IL-5 and IL-13 from granuloma associated cells, like eosinophils [45, 71-75]. IL-5 is essential in eliciting the granulomatous response because it is an important source of Th2 cytokines and contributes to the polarization of the immune response by recruiting eosinophils [74, 76]. IL-4-deficient mice are unable to mount an efficient Th2 response and develop an extremely polarized Th1 response that leads to 100% mortality during the acute phase [64, 77]. Moreover, in a Tamoxifen-induced IL-4 receptor α (IL-4Rα)-deficient mice are unable to mount an effective Th2 response which resulted in severe schistosomiasis and premature death [78]. IL-4 also enhances the effect of IL-13 on the development of fibrosis in a TGF-β independent manner [64, 79]. In *in vitro* studies, IL-13 has been demonstrated to stimulate collagen

production in fibroblasts and thus has a role in collagen homeostasis [80]. IL-4 and IL-13 are hypothesized to contribute to the development of fibrosis by inducing alternative activated macrophages (aaMΦ) [6, 66, 67]. aaMΦ promote collagen synthesis and fibrogenesis through the metabolism of L-arginine [6, 66, 67]. Since aaMΦ rapidly consumes L-arginine, other cells like T-cells are starved [81]. L-arginine is important for T-cell function as it modulates metabolic pathways in T-cells that contribute to cell survival and metabolic fitness [81]. Therefore, the Th2 response mediates the pathology due to the development of fibrosis during schistosomiasis.

The granulomatous response is regulated by several different cytokines to prevent host death and parasite survival. Eosinophil recruitment to granulomas, by IL-5, are crucial to disease progression but their exact role is undetermined [82]. The third T-helper cell subset (Th17) is not directly associated to the pathogenesis of schistosomiasis but elevated levels of IL-17 results in severe liver pathology after infection [83].

IL-10, a key regulatory cytokine, facilitates the shift from a Th1 to Th2 response which prevents the development of severe pathology from both responses [65]. Recently, Xiao *et al.* demonstrated that IL-10 expression is enhanced in splenic B cells during the chronic stage of *S. japonicum* which suppressed the function of Th1 and Th2 cells [84]. Regulatory T cells (Tregs), CD4+CD25+ subset of T-cells, inhibit the granulofibrotic response by suppressing DC activation and mediating the Th2 response [6]. This results in preventing extensive granuloma development and fibrosis. Interestingly, γδ T cells have been shown to secrete more Th2 cytokines, like IL-4 and IL-10, and fewer Th1 cytokines during lung-phase schistosome infection [85]. Natural occurring Tregs (nTregs) are recruited to the site of inflammation and suppress the expression of Th1 and Th2 cytokines [86]. The lack of Tregs during *S. mansoni* infection leads to increased weight loss, liver damage, and mortality [84]. Therefore, not only IL-10 and Tregs are necessary for host survival but they also play a crucial role in controlling schistosomiasis liver pathology.

The down-modulation of the Th2 driven granulomatous response is needed to prevent excessive inflammation and to prevent host morbidity [6]. This is characterized by the reduction of cellular inflammation caused by new egg deposits and the down regulation of Th2 cytokine levels [68, 86]. An important down-regulator of the granulomatous response and IL-13-mediated fibrosis is IL-13Rα2 [6]. It acts as a decoy receptor for IL-13, competing with IL-13Rα1, which prevents signaling through the IL-4/IL-13α1 receptor complex [65, 83, 87, 88]. Therefore, IL-13Rα1 and IL-13α2 expression may influence the severity of schistosomiasis [6]. Additionally,

apoptosis of CD4+ T cells have been suggested to contribute to the down-modulation of the granulomatous response [89]. *S. mansoni* infected Th2 skewed mice (C57BL/6) had smaller granulomas and higher rates of CD4+ T-cells compared to infected Th1 skewed mice (CBA) [89]. Furthermore, B-cell mediated FcR dependent signaling may also be involved in the down-modulation of the granulomatous response [90]. Mice that were deficient in B-lymphocytes or the Fc-receptor had exacerbated granuloma inflammation [90]. Therefore, the Th2 driven response to schistosome eggs in host tissue is necessary for host survival.

1.5 Immunomodulation by Schistosoma

The ability to survive and persist in a host is vital for parasite survival. When cercariae penetrate the host skin, they elicit a strong inflammatory response, therefore, making cercariae a prime target for immune cells. They quickly transform into migrating schistosomula. Specific cercarial molecules play immunomodulating roles such as smp_113760 (Sm16), a secreted cercarial protein with anti-inflammatory properties which is secreted upon penetration [91, 92]. SmDPP IV is primarily a membrane-bound protease. The exact role of SmDPP IV is unknown, however, homologues of it have been studied. An *Aspergillus fumigatus* homologue is involved in lung invasion and thus may facilitate schistosomula lung invasion [93]. Also, an mammalian homologue facilitates cell migration through connective tissue during wound repair which further supports the role of SmDPP IV in *Schistosoma* migration [94] . Cercarial elastase (SmCE) is a serine protease that not only comes from the acetabular glands of the cercariae but is also expressed when the cercariae are developing in the host snail [95]. SmCE has been shown to facilitate in human host invasion by *S. mansoni* cercariae [96, 97]. The shedding of the cercarial glycocalyx is vital to prevent massive complement fixation on the schistosomula surface [98, 99]. Moreover, secreted cercarial cysteine proteases have been shown *in vivo* to cleave host immunoglobins such as IgE and interfere with complement lysis [98, 99]. Sm16 also has pro-apoptotic properties once internalized by host cells [100]. Additionally, there are many proteins in cercarial secretions such as *S. mansoni* metalloprotease 8 (SmPepM8), SmDPP IV, and SmCE-1a, -1b, -2a [91, 101, 102]. SmPepM8 is a metalloprotease of the leishmanolysin family [103], The *Leishmania* homologue is involved in host invasion, therefore, it may have a role in cercarial invasion [101]. Cercarial proteases are essential in facilitating infection as they break down elastin in the host dermis to allow for parasite penetration [104, 105]. They are a group of serine-type proteases that are found

in pre- and post-acetabular glands of cercariae [106]. Cercariae also release lysophosphatidylserine which interacts with Toll-like receptor 2 (TLR-2) on dendritic cells (DCs) [99]. This allows for parasitic antigen presentation in a way that promotes the induction of Tregs and IL-10 production [99]. Lysophosphatidylserine also acts as a detergent and potentially lyses host cells, and thus could function as a mechanism of immune evasion [99]. The glycans found in cercarial secretions are high immunogenic and may act as a "smoke screen" to deflect antibodies away from other immunogenic peptides on the parasite to escape immune attack [107, 108]. Smp_194830 (SmKK7), secreted by the cercariae and schistosomula, is found in the nervous system in the adult worm [101]. It is a homologue to a component in scorpion venom that acts as a potassium ion channel blocker [101]. Moreover, when peripheral blood mononuclear cells (PBMCs) from *S. mansoni*-infected participants (prior to PZQ treatment) were exposed to recombinant schistosomula antigens lymphocyte antigen 6 isoform B (rSmLy6B), tetraspanin isoform (rSmTSP) 6, and rSMTSP7, strong Th1 and regulatory responses were elicited [109]. This suggests that the Th1 and regulatory responses are antigen-specific and thus can elicit a protective response in human resistance to reinfection [109]. Winkel *et al*, found that the cercarial secretions induce upregulation of programmed death-ligand 1/2 (PD-L1/2) and the program death-1 pathway to inhibit the adaptive immune response at the human dermis [110]. Additionally, they showed that excretory secretory molecules released by cercariae enhanced the production of IL-10, IL-6, and macrophage inflammatory protein-1α in the dermis after three hours of exposure [110].

Granuloma formation around the tissue-embedded parasite egg is vital for host survival. This Th2 driven response sequesters toxic molecules by the parasite egg [5]. The protective Th2 response is mediated by IL-4 as IL-4 deficient mice develop a severe IFN- γ induced inflammation and die prematurely [69, 111, 112]. aaMΦs promote collagen synthesis and fibrogenesis [6, 65, 66]. They have also been shown to facilitate wound healing and dampening Th2 inflammatory response [113, 114]. Additionally, Tregs are necessary to mediate Th2 responses, inhibit granuloma development, suppress DC activation and limit fibrosis [6]. For example, lysophosphatidylserine, a schistome derived molecule, has been shown to induce the production of IL-10 producing Tregs [115]. Since, IL-10 prevents the exacerbation of Th1 and Th2 responses, this molecule is important in allowing for parasite and host survival.

1.6 Snails: the intermediate hosts

It is important to consider the snail's immune response as it is a determinant of *Schistosoma* infection success. Snails and *Schistosoma* species have coevolved for over 200 million years [116]. Each *Schistosoma* species requires a specific snail intermediate host. In the case of *S. mansoni,* the snail host is *Biomphalaria* species whereas *S. japonicum* infects *Oncomelania* species, and *S. haematobium* miracidia infect the *Bulinus* snail genus [23]. The common disease model used to study snail immunity is *S. mansoni* infections in *Biomphalaria glabrata.* Haemocytes are the primary cells involved in anti-pathogen host reactions found in invertebrates [117]. Haemocytes are classified into two morphotypes, granulocytes and hyalinocytes [118, 119]. Granulocytes are more relevant in the anti-schistosome immune response as they are likely to come in contact with the sporocyst [119, 120]. Additionally, high levels of granulocytes (>230 cells/μL) in adult *B. glabrata* is associated with resistance to *S. mansoni* [121].

Humoral immunity is marked by the killing of schistosome sporocysts without encapsulation [117]. In particular, circulating humoral factors like fibrinogen-related proteins (FREPs) and biophlasin have been shown to facilitate *Schistosoma* death [122, 123]. Both humoral factors were transcriptionally upregulated during second challenge and the transfer of serum from primed snails to naïve snails decreased the prevalence of *S. mansoni* infections [122]. In invertebrates, recognizing invading pathogens is accomplished by thioester-containing protein (TEP) homologues [117]. TEP, in other invertebrate models, have been shown to bind to the α-2-macroglobulin (A2M) receptor on circulating phagocytic cells via their A2M receptor-binding domain [124, 125]. Moreover, *B. glabrata* thioester-containing protein (BgTEP) is associated with larval transformation products like *S. mansoni* polymorphic mucins (SmPoMucs) which suggests its role in the opsonization of *S. mansoni* sporocysts [126, 127]. Biomphalysin is a β-pore-forming toxin that has been shown to kill sporocysts in the presence of *B. glabrata* plasma [128-130]. Therefore, there is an undetermined cofactor that contributes to sporocysts killing mechanism employed by biomphalysin [117]. The role *B. glabrata* FREPs (BgFREPs) in the innate immune memory has yet to be described. Pinaud *et al.* demonstrated that the knockdown of BgFREP 2,3, and 4 reduced the innate immune memory phenotype by 15% [131]. Additionally, the group showed that BgFREP transcripts expression is increased during the secondary immune response of *S. mansoni* infection [131].

The cellular immune response against *S. mansoni* is characterized by the granulocyte recruitment to the site of infection [132]. Subsequently, a complete haemocyte multilayer around the schistosome sporocyst is formed, leading to death within 10-72 hours [119, 121]. The macrophage migration inhibitory factor is a haemocyte chemokine that facilitates granulocyte recruitment to sites of infection [133, 134]. Since schistosome sporocysts are too large to phagocytose, haemocytes strip sporocyst microvilli and small tegumental segments [119]. The transformation from miracidium-to-sporocyst causes the release of complex carbohydrates and glycoproteins [135]. The monosaccharides released have been shown to initiate high rates of phagocytosis in haemocytes mediated by BgFREP 3 [119, 135]. Moreover, FREPs and their interactions with *S. mansoni* polymorphic mucins (SmPoMucs) have been shown to be involved in parasite-host compatibility [136]. Larval transformation products have been shown to inhibit activity by interfering with cell motility, the capacity to produce reactive oxygen species (ROS), and intracellular protein synthesis of haemocytes in susceptible snails [137-139]. Additionally, schistosomes may release highly antigenic molecules that act as a "smoke screen" to dampen the snail's immune recognition [140]. Haemocyte activation, via *B. glabrata* pro-granulin cleavage, induces the production of cytotoxic materials such as hydrogen peroxide and nitric oxide, which have been shown to kill sporocyst [141, 142].

1.7 The Human Host

Since *Schistosoma* and humans have evolved together for centuries, some populations have developed natural immunities to protect against schistosomiasis like concomitant immunity, acquired immunity, and natural protective immunity.

In endemic regions, concomitant immunity prevents super infections of schistosomiasis. Concomitant immunity is characterized by effective anti-larval immunity and persistent adult worm infection [143]. Multiple studies have shown that adult schistosome worms alone are able to protect against reinfection [144-147]. Dissous and Capron identified a shared epitope between a 115-kDa adult worm molecule and 38-kDa schistosomula surface molecule [144]. Therefore, the presence of adult worms can induce the production of antibodies against schistosomula [144]. Additionally, with a single exposure of 250-500 *S. mansoni* ^{60}Co-irradiated cercaria as a vaccine in C57BL/6 mice demonstrated a 70-80% reduction in worm burden when challenged [148]. Therefore, the repeated exposure to cercaria contaminated water and parasite death in the host skin

may contribute to concomitant immunity [148]. Furthermore, Sombetzki *et al.* demonstrated that female-only adult worm infections suppress early innate immune responses to invading cercariae [144]. On the other hand, male-only infections trigger a strong innate immune response that leads to reduction in worm and egg burden in the liver [147]. Moreover, macrophages activated by adult worms have been shown to kill schistosomula *in vitro* [149]. Cardoso *et al.* demonstrated that high Sm29 IgG1 and IgG3 levels corresponded to individuals with natural resistance to infection and individuals resistant to re-infection post-PZQ treatment [150]. Lastly, TNFα levels are elevated in individuals with schistosomiasis and has been shown to be a larvicidal factor [149, 151].

Acquired immunity to schistosomiasis has seen in *Schistosoma*-endemic areas when exposed to schistosomes slowly over a period of 10 to 15 years [6, 152]. Therefore, adults have a lower prevalence of reinfection as they are more resistant to *Schistosoma* [152]. However, children under the age of 10 in these endemic regions are susceptible to reinfection after treatment [152]. Resistance to reinfection is linked to Th2 cell-associated responses by eosinophilia production of specific IgE against schistosomes and cytokines such as IL-4 and IL-5 [152]. Multiple studies have shown that specific IgE, eosinophils, and post-treatment production of IL-4 and IL-5 are associated with resistance to reinfection [153]. On the other hand, detection of specific IgG4 has been linked to susceptibility to reinfection [5]. A hypothesis is that the death of adult worms, either naturally or by PZQ treatment, leads to antigen release that may be cross-reactive to larval antigens and induce a protective IgE response [154]. Since the average adult worm's lifespan is 3-10 years, this can explain why adults have higher resistance to reinfection as they experienced many dying worms and thus have developed a protective immune response [155]. Since IL-10 levels are increased post-PZQ treatment, their involvement in schistosome resistance is of interest [156]. Wilson *et al.* showed that when PZQ treatment is coupled with IL-10 signaling blockage, it enhanced Th1, Th2, Th17 responses, worm-specific IgG1, IgG2b, and IgE, and increased the number of circulating eosinophils [156]. The inhibition of IL-10 signaling allowed for the development of protective immunity against reinfection with *S. mansoni* [156].

Natural protective immunity against schistosomiasis has been recorded in endemic regions such as Siqueira, Brazil. These individuals have not contracted schistosomiasis for over five years without anti-helminth drug treatment like PZQ [157]. Unlike other individuals infected with chronic schistosomiasis that develop a skewed Th2 response to schistosome antigens, endemic normal individuals (ENIs) have both Th1 and Th2 responses to parasite antigens [157].

Furthermore, there are increased levels of IgE specific for schistosomula surface antigens in ENIs compared to individuals with acquired immunity [158]. This suggests that IgE antibodies in ENIs target earlier stages of infection. Importantly, CD4+ T cells, from ENIs, secrete high levels of IFN-γ when stimulated with schistosome antigens [159]. It is hypothesized that IFN-γ triggers the lung phase immune response [159-161]. It is important to study the complex immunological relationship between schistosomes and their human host for the development of future therapeutics and vaccines.

1.8 Diagnostics

There are a number of different microscopy, serological, and molecular methods that are used to diagnosis schistosomiasis globally. Since labor-intensive methods are the main diagnostic methods of several diagnostic laboratories, there is a need for more rapid tests that can be used in clinical settings and be transported to remote, endemic regions [162].

One of the standard methods of diagnosing helminth infections is by direct examination of a parasite structure by microscopy [162]. Urinary schistosomiasis is diagnosed by microscopically analyzing urine samples for parasite eggs [162]. Ten milliliters of a mid-day urine sample are passed through a syringe with a polycarbonate filter (pore size of 8 μm – 30 μm) [163]. The parasite eggs are trapped by the filter, stained with Lugol's iodine, and then quantified by microscopy [163]. Similarly, intestinal schistosomiasis is by observing parasite eggs in stool with the Kato-Katz technique [164]. The thick smear Kato-Katz method involves sieving the stool, staining the sample, and analyzing it under a microscope [164]. However, these techniques are unable to detect low levels of infection and early stages of schistosomiasis [162]. Despite this, the Kato-Katz and urine filtration techniques are still widely used due to their simplicity and low cost.

Molecular methods to detect *Schistosoma* can improve the sensitivity and specificity in diagnostic assays. Polymerase chain reaction (PCR) with specific primers have been used detect *S. mansoni* in fecal samples with a detection limit of 2 eggs/gram of stool [42]. A real-time PCR (rt -PCR) with primers that targeted the small subunit rRNA of *S. mansoni* by Gomes *et al.* [165]. This method had a detection limit of 10 femtogram (fg) of purified genomic DNA which is equivalent to less than the DNA in one parasite cell [165].

Serological methods may allow for faster diagnosis and can provide additional insight. Serology-based diagnostic assays are typically used to test suspected imported cases of

schistosomiasis as microscopy alone may not be sensitive enough [166]. Although there are a few commercially available antibody detection assays, they are not standardized across laboratories. enzyme-linked immunosorbent assay (ELISA) uses soluble *S. mansoni* egg antigen (SWAP) [166]. The indirect hemagglutination (IHA) method uses erythrocytes that are coated with *S. mansoni* worm antigens and serum from patients with confirmed schistosomiasis by microscopy [166]. For detecting *S. mansoni* and *S. haematobium,* IHA has a specificity of 94.7% and a sensitivity of 86% [166]. And the specificity and sensitivity of ELISA assay are 97.2% and 98.2%, respectively. When combined, both the specificity and sensitivity of the assay were 97.2% [166]. The use up-converting phosphor-lateral flow (UCP-LF) reporter diagnosis procedure detects low-levels of parasite-excreted circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) in serum or urine (116-118). Importantly, UCP-LF can detect single worm infections [167, 168]. In Brazil, Sousa *et al.* compared UCP-LF CAA, SWAP- ELISA, PCR, and Kato-Katz techniques to diagnose *S. mansoni* [169]. The group found that the highest sensitivity tests were the urine CAA (80%) and serum CAA assays (70.9%) [169]. Additionally, the sensitivities of the other techniques were lower, SWAP-ELISA (43.6%), PCR (34.5%), and Kato-Katz thick smear (3.6%) [169]. Therefore, pairing serological and microscopy tests is needed to prevent underestimation of infection prevalence.

Recently, studies have demonstrated that extracellular vesicle products can potentially serve as stage-predictive biomarkers of schistosomiasis [170, 171]. In 2017, Meningher *et al*., compared serum EV RNA extracts between patients that were tested positive with schistosomiasis and healthy controls [170]. Interestingly, two schistosomal microRNAs (miRNA) , bantam and miR-2c-3p, were highly expressed during active schistosomiasis and had the sensitivity of 86% and specificity of 84%, [170]. They also tested seven patients post-treatment and found that a reduction in schistosomal miRNA was correlated to a full recovery [170]. Furthermore, the hepatic fibrosis stage of *S. japonicum* chronic infection can be graded with the detection of miR-146a-5p, a serum exosome derived miRNA [171]. By analyzing serum miR-14a-5p levels from patients with *S. japonicum* infection, researchers were able to distinguish between mild, grades 0-I, and severe, grades II-III, fibrosis with moderate accuracy [171].

1.9 Treatment

Praziquantel (PZQ) is the best treatment and morbidity control of schistosomiasis [19]. PZQ is a safe, effective, and cheap drug with a cure rate up to 85-90% where 100% cure rates can be seen in imported schistosomiasis cases but rarely recorded in endemic regions [172, 173]. The MDA of PZQ to school-aged children is one of the WHO's main methods of controlling morbidity rates of schistosomiasis [1]. School-aged children are at highest risk and it takes advantage of the educational infrastructure [174]. The standard PZQ dosage is 40 mg/kg for all ages and species [1]. PZQ is distributed as tablets that are taken orally [1]. Specifically, PZQ only eliminates the adult worm. PZQ does not kill immature worms present in the body at the time of treatment, prevent reinfection. and remove tissue-imbedded eggs [1]. Additionally, many field cases have shown that PZQ efficacy reduces with increased exposure to PZQ MDAs [175-178]. PZQ resistance can also be induced in controlled lab settings [179]. The exact mechanism of action is unknown and there is no test for praziquantel resistance [5]. There has been extensive research in how exactly PZQ affects the adult worm. Currently the early effects of the drug are known, and they are: (1) calcium influx into the whole parasite, (2) muscle contractions, and (3) surface modifications [20].

1.10 Schistosomiasis Vaccines

Since PZQ does not prevent re-infection or remove the host tissue-resident eggs, a vaccine against *Schistosoma* spp is important for disease control. In the past few decades, over 100 *Schistosoma* antigens have been analyzed for their protective efficacy [153, 180-182]. Many of the proteins studied for their potential vaccine are released by the parasite or on the parasite-host interface, [180].

1.10.1 SmCB

Schistosoma mansoni Cathepsin B (SmCB), an abundant cysteine protease used in Ndao laboratory, is found in the schistosomula and adult worm gut as well as somatic extracts. When SmCB transcription is suppressed with RNA interference, the parasite worms showed significant growth retardation compared to the control group [183]. Riccardi *et al.* demonstrated that when SmCB was administered with CpG dinucleotides and Montanide ISA 720 VG (squalene-based adjuvant), the vaccine reduced worm parasite burden by 54-59% and 56-62%, respectively [183, 184]. Importantly, SmCB with Montanide ISA 720 VG elicited a mixed Th1/Th2 response which

was characterized by elevated levels of IgG1, IFNγ,TNF-α, IL-4, and IL-5 [183]. Comparatively, SmCB with CpG immunization elicited a Th1 skewed response with elevated levels of IgG2c, IFNγ, and TNF-α [184]. Recently in the same lab, Perera *et al.* showed that when SmCB is administered with sulfated lactosyl archaeol (SLA) archaeosomes or AddaVax^{TM} (squalene-based oil-in-water emulsion adjuvant), both reduced granuloma size, and parasite pathology to the liver [185]. When SmCB is administered with SLA, the vaccine reduced adult worm, liver eggs, and intestinal eggs by 60.5%, 49.8%, and 59.4%, respectively, whereas AddaVax^{TM} reached 86.8%, 78.0%, and 83.4% in the same readouts [185]. The SmCB vaccine with SLA broadly elicited a Th1 mediated response. On the other hand, when paired with AddaVax^{TM}, the vaccine led to an increase in Th17 and Th2 cytokine production [185]. Another novel SmCB based vaccine by Hassan *et al.* is administered via an attenuated *Salmonella enterica* Typhimurium strain (YS1646) that expresses SmCB, through promoter-type 3 secretory signal pairs, to elicit a mucosal and systemic response [186]. The group demonstrated that when SmCB expressing YS1646 was administered orally with SmCB intramuscular vaccination, it reduced worm, liver eggs, and intestinal eggs, 93.1%, 79.5%, and 90.3%, respectively [186]. Moreover, this vaccine regime reduced granuloma size and decreased parasite egg fitness [186].

1.11 Exosomes and extracellular vesicles (EVs)

1.11.1 Exosome and multivesicular bodies biogenesis and secretion

Currently, multivesicular bodies (MVBs), ranging from 100 - 1000 nm, are used interchangeably with exosomes, ranging from 40 - 150 nm [187, 188]. MVBs are formed in secreting cells in endosomal compartments during a process called reverse vesicular invagination [188]. Exosome vesicular cargo is derived from the proteins processed and packed into MVBs [189]. The biogenesis of exosomes is unique from other extracellular vesicles, such as microvesicles (MVs) and apoptotic bodies. Exosome secretion in cell line models have been shown to decrease with chemical inhibitors of sphingomyelinase, i.e.: Na^+/H^+ and Na^+/Ca^{2+} channels, or H^+ pump [188, 190-193]. However, these inhibitors can induce major non-exosome-related issues as they act on molecules that are involved in multiple cellular pathways [187]. Additionally, exosome packaging and secretion mechanisms have been studied extensively. However, the extent of the molecular similarity between the packaged and secreted exosomes to their parental cells and whether they carry instructions to the potential recipient cells have yet to be described [194].

The endosomal sorting complexes required for transport (ESCRT) 0, I, II, and III are regulatory components involved in the transportation of ubiquitinated proteins into the MVB pathway [105, 106, 150, 195, 196]. The formation of the intraluminal vesicles (ILVs) of MVBs, therefore, involves the silencing and degradation of the endocytosed receptors where the cytosolic tails of the receptors are ubiquinated [197-200]. ILVs have two pathways, either they are delivered and degraded in the lysosome or they fuse with the plasma membrane wall and are secreted extracellularly as exosomes [194]. MVB-lysosome fusion, and consequent reduced exosome secretion, was demonstrated by cell exposure to type 1 interferons which caused the conjugation of proteins to the ubiquitin-like molecule interferon stimulating gene 15 (ISG15) [201]. However, ISG15 targets Tsg101, a component of the ESCRT machinery Tsg101/Vps23, involved in exosome secretion [201, 202]. Therefore, enhanced conjugation of ISG15 on ESCRT machinery may not be exosome exclusive [194]. Additionally, ESCRT machinery members, Tsg101/Vps23 and AIP1/ALIX/Vps31, have been shown to be essential in dendritic cell exosome secretion [203]. Also, the depletion of ESCRT-0 component hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) or signal transducing adaptor molecule 1 (STAM1) resulted in a decreased secretion of EVs with CD63 and MHC class II molecules, characteristic markers of exosomes [204]. Furthermore, when HRS is depleted during HIV infection, viral release from the plasma membrane is limited because tetherin degradation is prevented [205]. Since, MVB-derived exosomes also require tetherin to remain on the plasma membrane, it is suggested HRS is required for the secretion of both exosomes and plasma membrane-derived EVs [194, 206].

Transmembrane protein targeting ILVs of MVBs occur through several protein-protein interactions. ALIX, along with the ESCRT-I-III components, recruits syntenin-1 binding to the cytosolic domain of syndecan-1, and allows for inward budding into ILVs [207]. This mechanism relies on Src-mediated syndecan-1 endocytosis, phospholipase D_2 (PLD2), and ADP-ribosylation factor 6 (ARF6) GTPase activities [208, 209].

Several proteins are also involved in facilitating the ESCRT-independent mechanism of exosome biogenesis [210]. For example, tetraspanins such as CD9, CD63, and CD81 are transmembrane proteins involved in cell fusion, migration, and cell adhesion [210, 211]. The tetraspanin-enriched domain is the region where tetraspanins interact with other proteins, cholesterol, and gangliosides [210]. Importantly, the tetraspanin-enriched domains are involved in membrane binding and actin polymerization [210]. The study of exosome biogenesis in MVBs is

still actively investigated. Importantly, all known biogenesis mechanisms are not entirely specific to exosomes and are not valid in all cell types [210].

Moreover, pH has been shown to either trigger MVB content secretion or degradation [194].Degradation and recycling of internalized cellular components from MVBs is characterized by progressive acidification [212]. The dissociation of the E1 subunit of V1-ATPase by autophagy protein 5 (Atg5) and Atg16L1 abolishes its proton transporter activity and disrupts late-stage autophagy, thus reducing MVB acidification [212]. This has been shown to increase exosome secretion in human cells. On the other hand, reduced endocytosis and degradation of secreted exosomes, and indirect effects of disrupted late-stage autophagy on Golgi trafficking in cells also increases exosome secretion [213].

1.11.2 Exosome Cargo

Over the last decade, several -omics studies have clearly shown that exosomes do contain different types of biological macromolecules like lipids, nucleic acids, and soluble or membrane-bound proteins that maintain their activity when delivered to target cells. Exosomes have been shown to be mediators of cell-to-cell communication and can modify the behaviour of target cells [214]

1.11.2.1 Proteins

There are a variety of parent cell derived proteins that can be found in exosomes such as cytosolic, nuclear, mitochondrial, ribosomal, and membrane-bound proteins [210]. Through several proteomic studies of exosome proteins, some proteins are considered to be “exosomal markers” regardless of their cell origin. Vesicle-specific proteins are often used as markers such as cytosolic proteins (14-3-3 proteins), heat stress proteins (HSPs), tetraspanins (CD9, CD63,CD81), lectins, GTPases, major histocompatibility complex (MHC) molecules, and ESCRT complexes (Alix and TSG101) [211, 215, 216]. On the contrary, other exosome proteins are specifically related to the producing cell and can determine the exosomal properties and activities proteins [211]. According to a tumor-derived exosome (TDE) study, protein contents have the ability to regulate cell survival, tumor progression, metastasis, and chemoresistance [215]. Moreover, modifying integrin compositions of TDEs differentially drives them to specific tissues which induces an organ-specific pre-metastatic niche formation [217]. Many studies have shown that protein composition of exosomes is dependent on cell type and cellular conditions. For example, the protein content of chronic myeloid leukemia cell exosomes have depleted anti-angiogenic activity compared to untreated cells [218]. Since only a selected set of specific proteins

is packaged into exosomes, it suggests that there is a sorting system driven by specific mechanisms [211]. Several proteins isolated in exosomes have post-translational modifications like glycosylation, phosphorylation, ubiquitination, or SUMOylation [219].

1.11.2.2 Genetic material

Nucleic acids such as single-stranded (ssDNA) and double-stranded DNA (dsDNA), mitochondrial DNA (mtDNA), mRNA, micro RNA (miRNA), and long non-coding RNA (lncRNA) can be found in exosomes [214]. Exosomal DNA (exoDNA), genomic and mitochondrial, has been widely studied in many cell types [219]. Through the study of normal recipient human neutrophils and the transfer of BCR/ABL hybrid gene from K562 cells, exosomes are able to mediate the horizontal transfer of DNA [220]. The physiological significant of DNA exosome-mediated transfer is still not fully understood, however there is evidence demonstrating that recipient cells can localize exoDNA to the nucleus where it is transcribed [221]. Through the secretion of DNA fragments, the accumulation of cytoplasmic DNA does not occur and thus reduces cellular senescence or apoptosis [221]. Interestingly, exoDNA reflects the mutational status of parental tumor cells for genes such as p53, KRAS, and EGFR [222-225]. Therefore, exoDNA may be used as a diagnostic technique [222-224]. The exact mechanism that facilitates DNA loading into exosomes is still under debate [211]. One theory is that the DNA packaged into exosomes is dynamically regulated by cell type-specific mechanisms [222, 226]. Another hypothesis suggests that the presence of exoDNA fragments is equally distributed over the entire genome without bias for specific regions and thus is randomized [222, 224].

Exosomes contain RNAs , such as coding mRNAs and non-coding RNAs (miRNAs, lncRNA, rRNA, circular RNAs (circRNAs) are found in all cell types [227]. Several studies have shown that there is selective packaging of RNA molecules within exosomes [226-228]. Moreover, there is great interest in miRNA in exosomes because miRNAs are key regulators of gene expression [197]. Importantly, the miRNA profiles of parent cells differ from the secreted exosomes and thus their sorting into exosomes cannot occur randomly [229, 230].Current studies have shown that exosomal miRNA is guided by a specific sequence of miRNA and their interaction with certain enzymes and proteins [214, 231]. RNA-binding proteins are involved in regulating exosomal miRNA profiles [211]. Some of these proteins include the Y-box protein I (YBX1) has been shown to be required for the sorting of some exosomal miRNAs [232]. Moreover, ubiquitous

heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) has been shown to bind to miRNA EXO-motif and SYNCRIP protein is involved in miRNA sorting into hepatocyte exosomes [233, 234]. Another regulator of miRNA sorting into EVs are post-transcriptional modifications [211]. For example, Koppers-Lalic *et al.* showed that non-templated nucleotide addition 3'- urydilation on miRNA have been shown to be enriched in EVs and 3'adenylation miRNA have been reported in the origin cell [228]. Moreover, mRNA-miRNA interactions may modulate miRNA EV incorporation as miRNA is retained in the cytoplasm when expression levels of its target mRNA transcript are high [234]. Additionally, exosomes are stable in blood, urine, and other bodily fluids and they are a consistent source for miRNA and lncRNAs which can serve as disease biomarkers [211]. Therefore, the detection of exosomal RNAs is another method that allows for early detection, diagnosis, and clinical management of patients [211].

1.11.3 Exosome mechanism of action

1.11.3.1 Exosome interactions with recipient cells

Exosomes have been shown to regulate physiological and pathological states of their target cells through the horizontal transfer of their contents [211]. Therefore, exosomes are play a crucial role in cell-cell communication. Exosomes can alter cellular pathways through their internalization by recipient cells [211. There are two main mechanisms that are involved in the internalization of exosomes: receptor-mediated endocytosis and non-classical endocytosis. For receptor-mediated endocytosis, appropriate binding between an exosome surface ligand and its specific receptor on the recipient cell needs to occur [Conigliaro, 2020 #242. The interaction between the ligand and receptor triggers the assembly of coat proteins on the inner surface of the receiving cell. This facilitates the formation of clathrin-coated vesicles which detach from the cell membrane and continue in the clathrin-mediated endocytosis pathway [Kaksonen, 2018 #194, 235]. Furthermore, it has been demonstrated by Tian *et al.* that exosome internalization is inhibited by depleting clathrin with amphipathic drug treatment recipient cells [235]. The other mechanism for exosome internalization is non-classical endocytosis. This pathway is mediated by a number of different lipids in the plasma membrane such as lipid rafts, cholesterol, and sphingolipid-rich microdomains [211]. Another way that exosomes can be internalized is through phagocytosis by professional phagocytes and other immune cells like macrophages and dendritic cells [211]. However, endocytosis and phagocytosis drive the same pathway, internalized cargo to endosomal-lysosomal

degrative pathway [211]. The mechanism that internalized exosomes use to avoid lysosomal degradation is still unknown. A proposed strategy by Santos *et al.* suggest that a tripartite complex called VOR, composed of vesicle-associated membrane protein (VAMP)-associated protein A (VAP-A), cytoplasmic oxysterol-binding protein–related protein 3 (ORP3), and Rab7, drive late endosomes to the nuclear pore and then to the nucleoplasm [236]. The group showed that when CAP-A or ORP3 were silenced, the association of Rab7-positive late endosomes with nuclear envelope invagination was interrupted [236]. Therefore, EVs endocytosed by recipient cells, in this case mesenchymal stromal cells and breast cancer cells, were not transported to the nucleoplasm [236].

Exosomes do not interact with all cell types. They are selective couriers that preferentially bind to specific cell types [211]. For example, Hoshino *et al.* have demonstrated that only a few tissue-resident stromal cells uptake tumor exosomes and this uptake is dependent on the expression of integrins on the exosomes [231]. Exosome ligands interact with specific cell membrane receptors and activate downstream signaling pathways which leads to membrane fusion, internalization, and nuclear translocation of exosomal contents [211]. TDEs express death signals such as PD-L1 or Fas ligand (FasL) on their surfaces and thus promotes the suppression of the immune system by inducing apoptosis in receiving T cells and NK cells [237, 238]. Additionally, exosomes released by dendritic cells transduced with adenoviral vectors expressing IL-10, IL-4, or FasL are anti-inflammatory [239]. Exosomes have cell membrane ligands and receptors on their surfaces which facilitate cell-cell interactions that lead to downstream development, organogenesis, and tissue homeostasis [240]. For example, endothelial cell exosomes have been shown to have exposed Notch ligand D114 on their surface and thus promotes Notch-cleavage and activation when they encounter receiving cells [241]. Moreover, the D114 protein can be transferred to other endothelial cells and incorporated into their cell membranes [241]. This results in an inhibition of Notch signaling [241]. Importantly, this decrease of Notch signaling enhances vesicle formation [241].

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Exosomes affect recipient cells via their cargo. They have been shown to transform these cells and regulate tissue-specific and whole-body metabolism [211]. For example, Song *et al.* demonstrated that exosomes derived from cancer cells are enriched in phosphorylated receptor tyrosine kinases (RTKs) like human epidermal growth factor receptor 2 (HER-2) [242]. Once these exosome-derived phosphorylated RTKs are in tumor-associated monocytes, they will activate the MAPK pathway and increase monocyte survival [242]. Moreover, exosomes have been shown to amplify hormone signaling. Greening *et al.* demonstrated that exosomes derived from endometrial epithelial cells are essential in establishing pregnancy especially during the implantation stage [243]. The exosomes internalized by human trophoblast cells will enhance their adhesive capacity which contributes to the endometrial embryo interactions within the uterine microenvironment [243]. Furthermore, mesenchymal stem cells (MSCs) exosomes have been shown to be involved in tissue homeostasis [155]. These exosomes contain enzymes that are involved in restoring

glycolytic deficits and ATP production, growth factors involved in tissue repair like fibroblast growth factor and epidermal growth factor, and bioactive molecules that are involved in anti-inflammatory responses [155]. Therefore, MSC-derived exosomes have the potential to be used as therapies for various liver diseases such as, liver fibrosis, acute liver injury, and hepatocellular carcinoma [242].

1.11.3.2 Extracellular vesicles and Leishmania

Leishmaniasis is a neglected tropical disease caused by protozoa in the *Leishmania* genus [Alvar, 2012 #447 [244]]. Leishmaniasis has different disease manifestations: (1) localized cutaneous leishmaniasis, (2) mucocutaneous leishmaniasis, (3) diffuse cutaneous leishmaniasis, and (4) visceral leishmaniasis [244]. It is estimated that 556 million people are at risk of contracting visceral leishmaniasis and 399 million people are at risk of contracting localized cutaneous leishmaniasis in endemic regions [244]. Silverman *et al*, were the first group to report EVs budding from the flagellar pocket and plasma membrane from *Leishmania infantum* promastigote phase using scanning electron microscopy [245]. Other studies have demonstrated that *Leishmania* EVs contain virulence factors such as GP63 and lipophosphoglycan (LPG) which suggest that EV cargo maybe involved in disease pathogenesis [246, 247]. *Leishmania* EVs have also been found in the supernatant of promastigotes cultures and in the lumen of the sand fly midgut, therefore these findings also support the idea that *Leishmania* EVs maybe involved in leishmaniasis progression [247]. Moreover, when C57BL/6 mice were treated with *L. donovoni* EVs then infected, the mice showed higher parasite burden and IL-10 levels, compared to mice not primed with EVs [245]. BALB/c mice treated once with *L. major* EVs showed a similar result [246]. However, when these mice were treated twice with 15 μg of *L. major* EVs, there was a prominent Th2 polarization and disease exacerbation [246]. Interestingly, when *L. major* EVs were injected with the parasite in BALB/c footpads, the lesions developed were enhanced and there was an increase in IL-17a expression [244]. This finding suggests that *Leishmania* EVs are involved in establishing parasitic infection and parasite survival [244]. Additionally, when *L. mexicana* promastigote EVs were exposed to the J774 mouse macrophage cell line, it promoted the phosphorylation of MAP kinases in the naive macrophages [248].

1.11.3.3 Extracellular vesicles and Schistosoma

The study of extracellular vesicles (EVs) in *Schistosoma* is a growing field as the evidence for their involvement in disease progression, parasite-parasite and host-parasite communication, and other important roles are being discovered [249]. Currently, most *Schistosoma* exosome and EV research has been focused on the adult and egg life cycle stages [250-257]. However, some groups have isolated schistosomula exosomes and have studied their protein and small non-coding RNA composition, and their effect on dermal DCs and monocyte-derived DCs [244, 258-260]. Therefore, the novel ideas of studying EVs released by *Schistosoma* sporocyst in their intermediate host, *Schistosoma* cercariae during their transformation phase, and schistosomula during their lung migration phase may further our understanding of parasite-host interactions.

S. mansoni and *S. japonicum* adult worms live in the mesenteric plexus of the portal system for up to 10 years. Therefore, the parasite adapts various immune evasion tactics to survive. In 2015, Sotillo *et al.* was the first group to isolate exosome-like EVs ranging from 50-130 nm in size [255]. Adult worms were obtained from infected BALB/c mice 8 weeks post-infection and incubated for seven days [255]. The EVs were isolated in the incubation supernatant [255]. Their proteomic analysis showed that the EVs contained many *S. mansoni* vaccine candidate proteins and potential virulence factors [255]. Kifle *et al.* isolated EVs from two distinct exosome populations depending on ultracentrifuge spin speeds, at 15,000 x g and 120,000 x g, from *S. mansoni* adult worms [250]. Where the 15K pellet was collected with ultracentrifugation at 15,000 x g and 120K pellet was collected at 120,000 x g [250). Moreover, the proteomic analysis showed that there were distinct and shared protein populations in each pellet which varied in function and belonged to various biological processes [Kifle, 2020 #187]. For example, the 120K pellet had proteins in the tetraticopepetide repeat and tetraspanin protein families, whereas these families were absent in the 15K pellet [250]. Also, both pellets contained proteins from the Ras, AAA domain, and immunoglobulin domain, families [250]. The same group showed that EV proteins derived from *S. japonicum,* 120k and 15k pellets, demonstrated protection in a heterologous challenge with *S. mansoni* [252]. Mekonnen *et al*. created recombinant versions of the abundant tetraspanins, Sh-TSP-2, MS3_09198, and MS3_01370, found in *S. japonicum* adult EVs at 120K and 15K pellets to formulate the vaccines [252]. Another group has isolated *S. mansoni* adult exosomes and analyzed their microRNA (miRNA) [254]. These miRNAs have been shown to be present at high levels in the sera of infected hosts [254]. This suggests that vesicle-mediated

secretion of parasite miRNA in exosomes may be used to develop vaccines and therapeutics as they may play a role in host-parasite communication [254].

Furthermore, proteomic analysis of EVs derived from *S. japonicum* adult worms demonstrated that most of the proteins identified were involved in binding, catalytic activity, and translation regulatory activity [256, 257]. Importantly, their studies also demonstrated that mammalian cells internalize *S. japonicum* EVs and transfer their exosomal miRNA to the host recipient cells [257]. The same group showed that the miRNA derived from *S. japonicum* EVs were able to regulate host macrophages to allow for parasite modulation of the host immune response to allow for parasite survival [251]. Recently, the proteomic analysis and deep sequencing of EVs from adult male and female *S. japonicum* demonstrated the enrichment of 18 miRNAs in male and female adult worm EVs [258]. In particular, there was an increase if miR-750 in female EVs [258]. The inhibition of miR-750 by a mRNA inhibitor resulted in a decrease in egg production in female adults cultured *in vitro* [258]. The results from this study imply that miR-750 derived from female adult *S. japonicum* EVs may regulate ovary development and egg production in the parasite [258]. The same group is in the preliminary stages of evaluating the diagnostic potential of *S. japonicum* EV (SjEV) proteins [259]. Chen *et al*. demonstrated that the sera from *S. japonicum* infected mice, rabbits, and humans had detectable antibody levels against two SjEV antigens [259].

Lastly, *S. japonicum* eggs are trapped in host tissues and are a major contributor to severe pathology of schistosomiasis. As described previously, adult derived EVs have been shown to play a role in parasite-host communications, therefore the role of egg derived EVs should also be investigated to determine their role in schistosomiasis. Zhu *et al.* cultured *S. japonicum* eggs *in vitro* and isolated and analyzed the EVs that were secreted [260]. Additionally, murine liver cells (Hepa1-6) cultured with schistosomal egg EVs *in vivo* and primary hepatocytes from *S. japonicum* infected mice were also analyzed [260]. The small non-coding RNA (sncRNA) populations in the hepatocytes were cross-referenced with the sncRNA populations found in schistosomal egg EVs [251]. In both models, two miRNAs, Sja-miR-71b and Sja-bantam, were shown to be found in the recipient cells [260]. Therefore parasite-derived miRNAs were transferred to recipient host cells via EVs [260].

Some groups have isolated and analyzed *S. mansoni* schistosomula derived EVs. Since the schistosomula stage is the first to successfully manipulate host defenses and allow for parasite

survival, the mechanisms behind host immune evasion are an important area of study. Norwackie *et al.* performed the first proteomic, miRNA, and tRNA-derived small RNAs (tsRNA) analysis of EVs from schistosomula cultured *in vitro* [259]. The group characterized a core set of 109 proteins, including homologs to proteins found in high levels in other eukaryotic EVs [253]. Moreover, ES sncRNAs from inside and outside the EVs identified 35 known and 170 potential gene-regulatory miRNAs, and 43 tsRNAs [253]. Winkel *et al*, found that the cercarial secretions alone were unable to stimulate DDCs and MoDCs [110]. Therefore, direct contact with the cercariae is required to induce upregulation of PD-L1/2 and the PD-1 pathway to inhibit the adaptive immune response at the human dermis [261]. In March 2020, Kuipers *et al*. discovered that dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN, CD209), is necessary for schistosomula EV internalization and consequent upregulation of MoDC activation [261]. The group also demonstrated that schistosomula EVs increased IL-12 and IL-10 expression by MoDCs, thus schistosomula EVs facilitate immunomodulation [261].

1.12 Rationale and Research Objectives

Many groups have investigated the contents and the roles of *Schistosoma* EVs, particularly those derived from adult worms and eggs. However, none have studied the contents and possible roles of EVs derived from *S. mansoni* cercariae. Since the cercariae life cycle stage is actively infecting the host through the skin, EVs secreted at the time of penetration may aid in understanding the host-parasite relationship further. Therefore, the high disease burden of schistosomiasis and the shortage of therapeutic and preventative agents against schistosomiasis justifies further research in understanding the host-parasite interface at the site of infection.

This leads us to our research question: What are the potential roles of *S. mansoni* EVs secreted at the time of infection?

In our research proposal, we hypothesize that "through the proteomic analysis of *S. mansoni* derived cercaria EVs, we can further understand host-parasite relationship at the site of infection." To determine the validity of our hypothesis, the following research objectives were proposed:

1. Develop an *in vitro* method where EVs secreted during *S. mansoni* infection can be isolated

2. Analyze the protein contents of the isolated EVs secreted by *S. mansoni* cercariae during infection

Chapter 2: Methods, Results, and Discussion

2.1 Materials and Methods

2.1.1 *Schistosoma mansoni* cercaria extracellular vesicle isolation

Biomphalaria glabrata snails infected with Puerto Rican of *Schistosoma mansoni* were provided by the National Institutes of Health–National Institute of Allergy and Infectious Diseases (NIH-NIAID) of the Biomedical Research Institute (Rockville, Maryland, USA). *B. glabrata* snails were under light for 1 hour in 28°C water to trigger the release of cercariae [262]. In our first sample, roughly 20,000 cercariae were collected and our second sample had around 90,000 cercariae collected. For the production of cercarial EVs, cercariae were heat-shocked with pre-warmed (37°C) RPMI 1640 without phenol red (Thermo Fisher Scientific, Rockford, IL, USA). The sudden change of temperature allows for cercariae to begin their transformation into schistosomula as it simulates cercariae entering the human host. Immediately, the cercariae and the media were transferred into a petri dish and incubated at 37°C and 5% CO_2 for 1.5 hours to aid in transformation and EV secretion. To remove parasites and other debris, the supernatant and parasites were spun with the Beckman Allegra 6R Refrigerated Centrifuge (Beckman Coulter Life Sciences, Indianapolis, IN, USA). The supernatant was aspirated leaving parasites and other debris at the bottom of the falcon tube. The mixture was spun twice at 300 x g for 10 minutes where the supernatant was collected. The supernatant was then filtered with a 0.45 μm syringe filter. The supernatant was then transferred into ultracentrifuge tubes (17 mL open-top thin wall polypropylene tube (16 x 102 mm) (Beckman Coulter Life Sciences, Indianapolis, IN, USA).

EVs were pooled in 17 mL thin-wall polypropylene tubes and pelleted by ultracentrifugation in a Beckman Optima^{TM} XPN-90 (Beckman Coulter, Brea, CA, USA) with an ultracentrifuge rotor, Beckman Coulter SW 32.1 Ti swinging-bucket (Beckman Coulter Life Sciences, Indianapolis, IN, USA). We spun our samples for 100,000 x g for 1 hour. The pellet was resuspended in exosome buffer (137 mM NaCl and 20 mM HEPES (pH 7.5)). To collect the EVs, the supernatant was then spun again at 100,000 x g for 1 hour. The pellet was collected for further analysis and stored at -80°C for future analysis.

For protocol optimization we tried different techniques to stimulate EV secretion by the cercariae. We tried involved different media types: DMEM and RPMI 1640 without phenol red supplemented with 5% fetal bovine serum and 200 μg/mL of streptomycin and penicillin (Thermo Fisher Scientific, Rockford, IL, USA). We also tried different transformation techniques prior to

parasite incubation. This was done by agitating the cercariae as a method of mechanical transformation by using a vortex on the samples for 30 seconds. Lastly, we experimented with different incubation times from 15 minutes to overnight at 37°C, 5% CO_2. We also tried adding a 0.22 μm syringe filter step after the 0.45 μm syringe filtration step. These steps were then followed by the same differential centrifugation and ultracentrifugation steps as stated above.

2.1.2 Characterization of Schistosoma mansoni cercaria extracellular vesicles

2.1.2.1 MicroBCA Protein Assay

EV samples were quantified with the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) according to kit instructions. The protein standards with bovine serum albumin (BSA) were diluted accordingly with exosome buffer. Samples were diluted 1:20 to achieve a final volume of 240 μL per sample in a 96 well plate (Thermo Fisher Scientific, Waltham, MA, USA). The plate was covered using sealing tape and incubated for 37˚C for 2 hours and then allowed to cool to room temperature. The plate was then read at 562 nm and a standard curve was created to calculate the protein concentrations of the EV samples.

2.1.2.2 Nanoparticle tracking with Nanosight

Samples were diluted with exosome buffer to a protein concentration of 2 μg/mL, based on the MicroBCA results. The total volume in the 1.5 mL microcentrifuge tube should be 1000 μL. The nanoparticle tracking device used was the Nanosight NS300 (Malvern Panalytical, Malvern, Worcestershire, UK). The NanoSight settings were set up by and the samples at the Dr. Janusz Rak lab at the Research Institute of the McGill University Health Centre.

2.1.2.3 Transmission electron microscopy

Transmission electron microscopy (TEM) was performed at the Facility for Electron Microscopy Research of McGill University. EVs were transferred to Carbon Films on 200 Mesh Grids Copper (Agar Scientific Ltd, Stansted, Essex, UK) with grid tweezers. EVs were fixed in 1% glutaraldehyde (Millipore Sigma, Burlington, MA, USA) and stained with 1% uranyl acetate. Samples were visualized using FEI Technai012 120 KV electron microscope.

2.1.2.4 Trichloroacetic acid protein precipitation

Samples were diluted with exosome buffer to 5 ng/µL based on MicroBCA results to a final volume of 100 µL. Equal parts of 10X Tris-HCl and EDTA (TE) buffer, 0.3% sodium deoxycholate (NaDoc), and 72% Trichloroacetic (TCA) were mixed with the sample and incubated on ice for one hour. The mixture was then centrifuged a 18,400 x g with the Eppendorf 5424 R (Eppendorf Canada, Mississauga, ON, Canada) at 4°C for 20 minutes. After aspiration, the pellet was resuspended at room temperature in 90% acetone. The sample was incubated overnight at -20°C. Then it was centrifuged at 18,400 x g at 4°C for 20 minutes and the supernatant was aspirated. Pellet was air dried and then stored at -80°C.

2.1.2.5 Liquid chromatography tandem-mass spectrometry

Liquid chromatography tandem-mass spectrometry (LC-MS/MS) was performed at the Institute de Recherches Cliniques de Montréal (IRCM, University of Montréal). The EVs were prepared with TCA precipitation for triplicates of 5 µg for each sample. Proteins were precipitated with 15% TCA/90% acetone and digested with trypsin at a final concentration of 2ng/µL. After an 18-hour incubation at 37°C, the samples were quenched by adding formic acid to a final concentration of 1% before LC-MS/MS. The LC column used was a ProFrit fused silica capillary column (New Objective, Littleton, MA, USA) self-packed with C-18 reverse-phase material (Phenomenex, Torrance, California USA). Next, the LC column was installed on the Easy-nLC II system (Proxeon Biosystems, Odense, Denmark) and coupled to the Q Exactive mass spectrometer (Thermo Fisher Scientific, Rockford, IL, USA) with a Proxeon nanoelectrospray Flex ion source. There were two buffers used for chromatography. Buffer A was 0.2% formic acid and buffer B was 100% acetonitrile with 0.2% formic acid. The proteins were loaded onto the column at a flow rate of 600nl/min and eluted with a two-slope gradient at flow rate of 250 nl/min. Solvent B is increased from 2% to 40% acetonitrile in 85 minutes then from 40% to 80% in 25 minutes. The data from LC-MS/MS was acquired using a data-dependent top 15 method and standard values were used for all standard mass spectrometer parameters.

2.1.2.6 Protein database search

All MS/MS samples were analyzed using Mascot (Matrix Science, Boston, MA, USA; Mascot in Proteome Discoverer 2.4.0.305). Mascot was set up to search UniProt_Schistosoma assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 parts per million (PPM). Carbamidomethyl of cysteine was used as the fixed modification in Mascot, and the oxidation of methionine was used as a variable modification in Mascot.

Scaffold software version 5.0.1 (Proteome Software, Portland, OR, USA) was used to validate MS/MS peptides and protein identification. Peptide identification was accepted if they had a greater than 80% probability. The identification of proteins was accepted if it had a 95% minimum probability and at least two identified peptides. Peptides that were also found in blanks were removed from analysis. Proteins were accepted if they were present in at least two of the six samples ran. Proteins that contained similar peptides but were not differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins that shared significant peptide evidence were grouped into clusters.

2.2 Results

2.2.1 Isolation and characterization of extracellular vesicles

Currently, most *Schistosoma* exosome and EV research has been focused on the adult and egg life cycle stages [250-257]. Therefore, the novel idea of studying EVs released by *Schistosoma* cercariae during their transformation phase may further our knowledge on parasite-host interactions during infection. Since infectious part of the *Schistoma* life cycle is the cercaria, investigating the exact mechanisms of action at the time of infection is important. Researching the infection process may aid us in developing therapeutics and vaccines for schistosomiasis. The isolation of cercarial EVs from *Schistosoma mansoni* has not been studied so an optimized protocol needed to be established to ensure a high yield of homologous EVs. We determined that the protocol that utilized heat induced cercarial transformation and an incubation period of 1-2 hours was ideal for increased parasite viability and EV secretion. During the incubation period, parasites were monitored their health through their mobility. Cercariae and schistosomula that were moving

slowly compared to normal were considered to be unhealthy. Thus, we ended the incubation periods as soon as we notice that approximately 10% of the parasites were becoming less motile. Additionally, it is important to note that cercariae and schistosomula death occurs during natural infection so EVs collected from dying cercariae/schistosomula are representative of a natural infection. After we isolated the EVs, we ran a MicroBCA for protein quantification. We found that the concentration of sample 1 (S1) was 113.11 µg/mL and that of sample 2 (S2) was 504.82 µg/mL. Therefore, the approximate amount of protein in our 300 µL samples were 1.41 µg in S1 and 6.3 µg in S2. Next, nanoparticle tracking (NTA) with Nanosight was required for particle concentration and estimation on the general size of the particles in our samples (Figure 1 and Table 1). We found that the mean diameter size of the particles was 184.4 nm and 153.4 nm in S1 and S2, respectively. The mode (the most frequent) diameter size of the particles in 131.3 nm and 121.1 nm in S1 and S2, respectively. The concentration in S1 and S2 were $1.95 \times 10^8 \pm 3.09 \times 10^7$ and $4.63 \times 10^8 \pm 2.11 \times 10^7$ particles/mL, respectively. Additionally, the estimated number of particles in our total samples were 5,559 and 22,165 for S1 and S2, respectively (Table 1). Lastly, to confirm the identity of our particles as EVs, we performed transmission electron microscopy (TEM). As seen in Figure 2, the EVs isolated were homologous in size and were roughly 100 nm in diameter. The EVs also had a distinct double membrane bilayer which is characteristic of exosomes (Figure 2).

During protocol optimization we tried different variations for high output of EVs without compromising parasite health. We tried different culturing medias like DMEM and RPMI 1640 without phenol red with supplementation (5% exosome depleted FBS and streptomycin/penicillin). In non-supplemented DMEM and RPMI 1640 without phenol red, we observed decreased mobility and viability in cercariae and schistosomula with incubations that ranged from 4 hours to 24 hours. Interestingly, there were extremely low concentrations of protein in the samples, below 20 µg/mL, and no EVs detected using TEM. Furthermore, EVs secreted during parasite death are not representative of EVs secreted during cercariae penetration. Therefore, we chose to add 5% exosome depleted FBS and antibiotics to RPMI 1640 and DMEM. When cercariae and schistosomula were cultured in supplemented media, the parasites were healthier as they were more motile. However, with TEM analysis, there were low concentrations of EVs and a high concentration of background proteins from the FBS (Appendix 4). Thus, we moved back to using RPMI 1640 without phenol red alone as parasites were slightly healthier in

this media opposed to DMEM. Next, we varied different incubation periods to combat the quick decline of cercariae and schistosomula health during incubation. We decided to decrease the incubation period from overnight to 1-2 hours. This allowed us to isolate EVs that were secreted during cercariae transformation into schistosomula without collecting EVs that may have been secreted due to parasite death. Additionally, we only used a 0.45 μm syringe filter. We hypothesized that the 0.22 μm filtration step may remove EVs that were aggregated during isolation. Thus, to maximize EVs isolated, we removed the finer filtration step. Lastly, we attempted to transform the cercariae into schistosomula in two different ways, mechanically with vigorous agitation and a heat shock method with 37°C media. We paired an incubation step of 4-hours or overnight incubation at 37 °C, 5% CO_2 with mechanical transformation as it simulates a natural infection. This resulted in many dead parasites and low levels of EVs according to MicroBCA, NTA, and TEM analysis (Appendix 5). The TEM image in appendix 5 was the only EV found in all of our samples. Thus, the coupling of mechanical and heat induced transformation was ineffective in producing EVs. Therefore, we moved forward with our experiments with heat shock only as it limited parasite death due to damage.

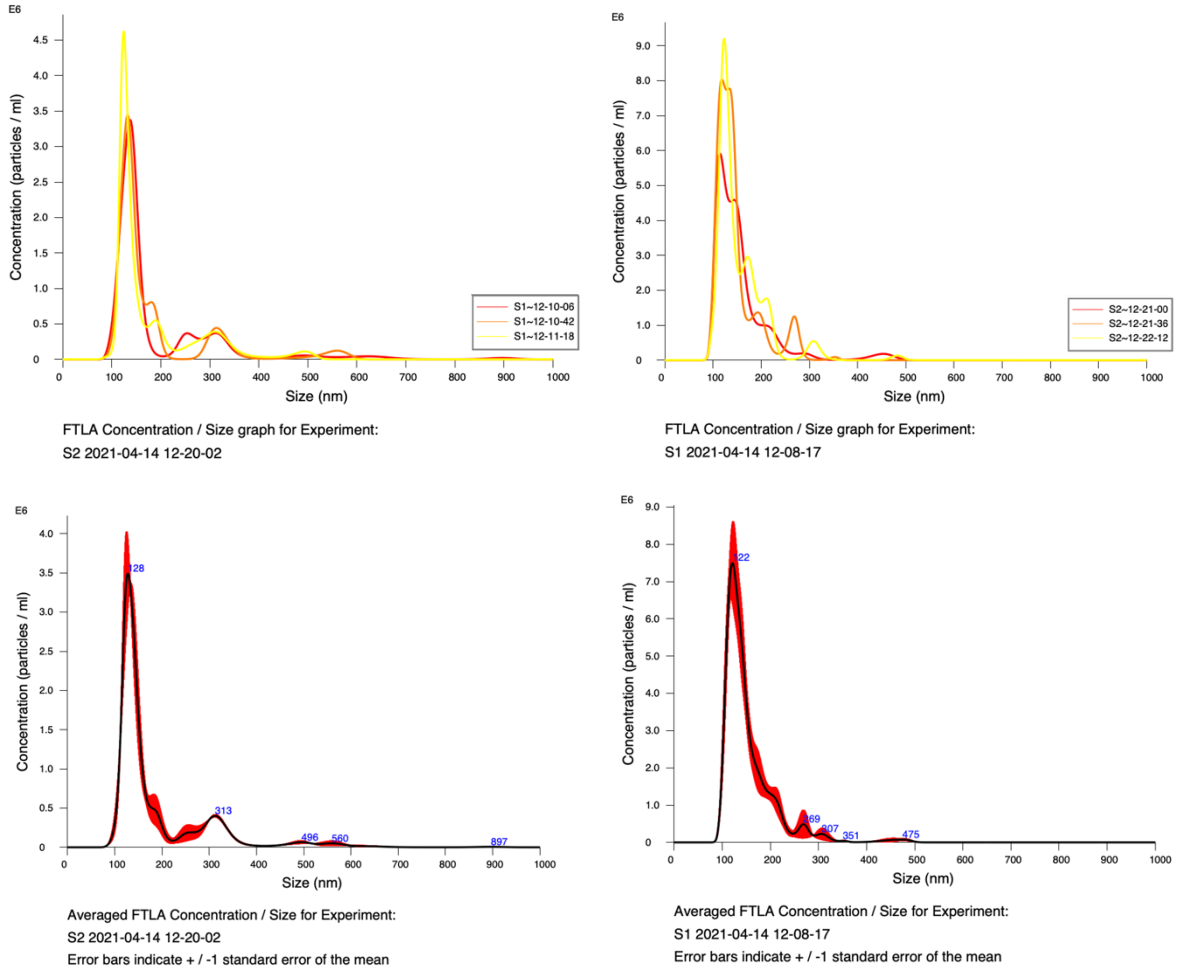

Figure 3: Nanoparticle tracking analysis of S. mansoni cercariae EVs

The analysis was performed from EVs suspension extracted from secretions from *S. mansoni* cercariae during in vivo heat shock to stimulate transformation into schistosomula. Representative Nanosight generated graphs of nanoparticle size distribution and concentration in particles per mL. On the top, each line represents the concentration in one video. On the bottom are the average concentrations calculated from the three videos taken. Graphs represent two different preparations.

Sample	Mean Size (nm)	Mode Size (nm)	Concentration (Particles/mL)	Estimated number of particles in 300 µL sample
S1	184.4	131.3	$1.95 \times 10^8 \pm 3.09 \times 10^7$	5,559
S2	153.4	121.1	$4.63 \times 10^8 \pm 2.11 \times 10^7$	24,165

Table 1 : Particle concentration and size of particles S. mansoni cercariae EVs from Nanosight

The analysis was performed from EVs suspension extracted from secretions from *S. mansoni* cercariae during in vivo heat shock to stimulate transformation into schistosomula. The data is derived from nanoparticle tracking analysis software Nanosight. The concentration, mean and mode sizes are the average of the three videos taken of EV suspensions. The estimated number of particles calculation did not take into account variance.

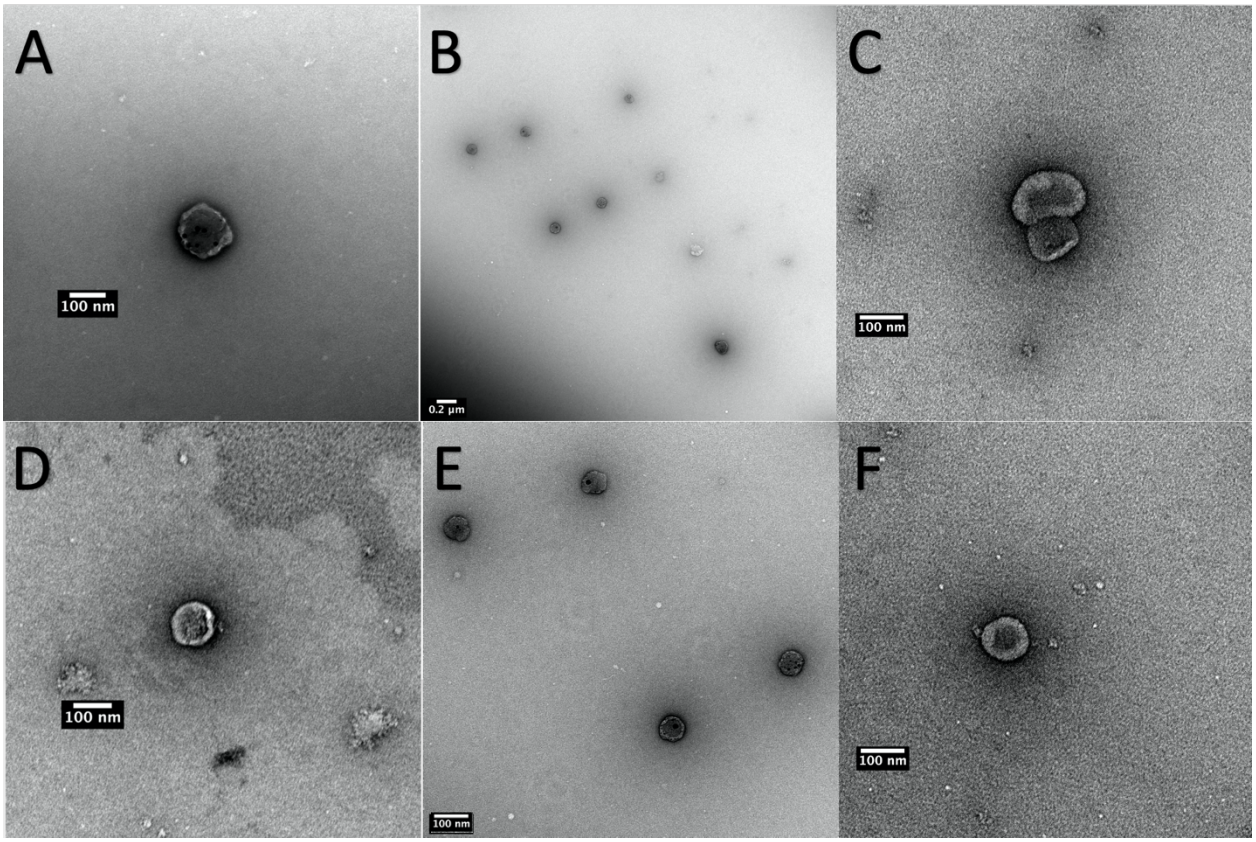

Figure 4: Transmission electron microscopy photos of S. mansoni cercariae EVs

The analysis was performed from EVs suspension extracted from secretions from *S. mansoni* cercariae during *in vivo* heat shock to stimulate transformation into schistosomula. The EVs were stained with uranyl acetate to visualize morphology. Images A, C, D, and F were taken at 32000x; scale bar represents 100 nm. Image B was taken at 9000x; scale bar represents 100 nm. Image E was taken at 13000x; scale bar represents 0.2 m.

2.2.2 Proteomic analysis of extracellular vesicles

LC-MS/MS was conducted on triplicates of two different preparations of purified vesicles from cercarial transformation vesicles and candidate proteins were identified via searching against the *S. mansoni* NCBI genome database. Based on our peptide and protein identification thresholds, we were able to identify 25 known *S. mansoni* proteins (Table 2). Interestingly, many of the proteins detected are common exosomal markers such as CD63, enolase, actin, tetraspanins, tubulins, 14-3-3 proteins, elongation factors, and heat shock protein 70 (HSP70) [263-265]. Therefore, the presence of these proteins supports that the vesicles isolated are exosomes.

Most proteins that were identified belonged in the signal transduction and biological regulation group according to GO biological processes terms (Table 2). For example, a 14-3-3 *S. mansoni* homolog was identified and this group of proteins, generally, is widely involved in many different general and specialized signaling pathways like protein synthesis and epithelial cell growth by Akt/mTOR pathway stimulation [266, 267]. Additionally, calmodulin is a protein involved in calcium-mediated signal transduction [268]. Calmodulin has also been shown to be involved in cellular processes that stimulate muscle contraction, calcium homeostasis, and inflammation [269]. We also identified an unidentified calcium binding protein which may also be involved in calcium-mediated signaling. Another protein identified was cGMP-dependent protein kinase. It is involved in protein serine/threonine kinase in ATP binding and phosphorylation. This group of kinases have been shown to initiate nitric oxide and atrial natriuretic peptide signaling cascades [79]. However, the roles of 14-3-3 proteins, calmodulin, and cGMP-dependent protein kinases have not been studied in *Schistosoma.* Thus, possible functions of these proteins have been derived from studies in humans and other mammals. Albumin was also identified in cercariae secreted EVs. *Schistosoma* albumin gene expression is induced when adult or schistosomula were exposed to oxidative stress in culture [79, 270]. Therefore, it was suggested that parasite albumin may neutralize oxidative assault generated by the host immune response [270, 271]. The EF-hand domain containing protein was unidentified. However, EF-hand domain containing tegument proteins have been demonstrated to assist schistosome escape from the host immune response through inhibiting chemotaxis and non-complement fixing antibody (IgG4) responses [272].

Other proteins that were identified were parts of the cell structure and cytoskeleton. Actin, tubulin α/β- chains, Rab-2B, Fer1 and tetraspanins like CD63 are found in the cellular membrane

and are crucial components for cell membrane integrity. Importantly, CD63 homolog was identified in our exosomes and it is a major marker for exosomes [265]. Additionally, CD63 and other tetraspanins are crucial for cell adhesion and migration [273]. CD63 homolog Sm-TSP-2 is a vaccine candidate as it was able to provide high levels of serum IgG that is protective against *S. mansoni* infection in humans [274]. Currently, a Sm-TSP-2 vaccine, Sm-TSP-2/Alhydrogel® vaccine is undergoing clinical trials [274]. Furthermore, Fer1 is preferentially expressed in female *S. mansoni* adult worms as it is present in the yolk platelets in eggs and thus aid in miracidia development [275, 276].

Cercarial proteases are essential in facilitating infection as they break down elastin in the host dermis to allow for parasite penetration [104, 105]. They are a group of serine-type proteases that are found in pre- and post-acetabular glands of cercariae [106]. We were able to identify *Schistosoma* vaccine candidate Sm29 [150]. Sm29 is a membrane-bound antigen and is abundantly found on the outer surface of the adult worm and schistosomula [195].

Metabolic proteins analysis identified serpin, enolase, and glycogenic-related protein in cercarial EVs. These proteins are vital for parasite survival. For example, serpin is a serine proteinase inhibitors that inhibits cercarial proteases through complex formation [196]. It is typically expressed in later schistosomula head glands and adult worm spines to modulate serine proteases in *Schistosoma* and their host [277, 278]. Additionally, *S. mansoni* enolase is a host-interactive tegumental enzyme that can bind to and enhance plasminogen activation to degrade blood clots to aid in parasite migration [277].

Lastly, three other proteins were identified which were also prominent in exosomes. Ubiquitin has been shown to be enriched in exosomes compared to total cell lysates and have been hypothesized to be involved in sorting proteins into exosomes [279]. Elongation factor 1-α mainly functions as a protein synthesis regulator [280, 281]. HSP70 is a chaperone that is regulated by stress. Therefore, it is highly expressed during the cercariae-schistosomula transformation [282]. Generally, HSP70 activates the innate immune system independently without binding to the antigenic peptides [283]. It has also been shown to induce the release of pro-inflammatory cytokines from innate immune cells [283].

Since cercariae release their pre-acetabular glands at the time of penetration in addition to EV release, it is important to note differences between their proteomic profiles. As seen in table 2, we found four overlapping identified proteins, 14-3-3 homolog, HSP70, cercarial protease, and

elongation factor 1-α, that were also found during cercariae-schistosomula mechanical transformation and skin-invasion *ex-vivo* [102, 284]. Therefore, most of the proteins we were able to identify are found only in the EVs we isolated from *S. mansoni* cercariae-schistosomula transformation.

It is also important to note that there were low levels of protein in our proteomic samples most likely due to disturbing the pellet during TCA protein precipitation. Therefore, proteins that were present in one of the six samples ran could be in our EVs, but low levels of protein prevented their detection. As seen in A2 in the appendix, all the proteins identified belong to *Schistosoma*. Some notable proteins include venom allergen-like 16 (VAL16), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) , and ATP-diphosphohydrolase 1. VAL proteins have been shown to be highly expressed during cercarial invasion and VAL4 has been found in human skin post invasion [284, 285]. Also, VAL1, 2, 16, 17, and 21 has been shown to be highly expressed during the cercariae stage [286]. Next, GAPDH is a candidate subunit vaccine that induces protective immunity against *S. mansoni* when administered with cysteine peptidases [287]. Ridi *et al.* demonstrated that recombinant GAPDH elicited a protective T- and B-cell response which correlated to resistance to *S. mansoni* reinfection post PZQ treatment [288]. Additionally, ATP-diphosphohydrolase 1 (ATPDase-1) has been shown to suppresses inflammation and inhibits platelet aggregation [289].

Proteins identified	Accession Number	Secreted by Cercariae/Schistosomula reference
Cytoskeletal/Structural Proteins		
Actin-1	**A0A4Z2DUG9_SCHJA**	
Tubulin alpha chain	G4V865_SCHMA	
Tubulin beta chain	G4VTA5_SCHMA	
RAS-like GTP-binding protein	C1L8G6_SCHJA	
Cluster of Putative rab15, 13, 10, 1, 35,and 5	A0A3Q0KV72_SCHMA	
Putative rab-2,4,14	G4VRU0_SCHMA	
Tetraspanin	**A0A5K4F3T7_SCHMA**	
Putative tetraspanin-CD63 receptor	**A0A3Q0KN99_SCHMA**	
Fer-1-related	A0A3Q0KNB2_SCHMA	
Metabolic		
Enolase	**ENO_SCHMA**	
Serpin, putative	G4LZN6_SCHMA	
Glycogenin-related	A0A3Q0KC44_SCHMA	
ATP-dependent RNA helicase	A0A430PXQ6_SCHBO	
Translational		
Elongation factor 1-alpha	**A0A5K4F1Y4_SCHMA**	115
Signal transduction and biological regulation		
cGMP-dependent protein kinase	A0A3Q0KI04_SCHMA	
14-3-3 protein homolog 1	**14331_SCHMA**	115
Calmodulin	E9LZR7_SCHMA	
Calcium-binding protein	CABP_SCHMA	
EF-hand domain-containing protein	A0A183QE06_9TREM	
Albumin	Q95VB7_SCHMA	
Protease		
Cercarial protease	CERC_SCHMA	275
Tegumental antigen		
Sm29*	A0A3Q0KHJ2_SCHMA	
Chaperone		
Putative heat shock protein 70	**A0A5K4F5D3_SCHMA**	275
Other		
Polyubiquitin-C (Fragment)	A0A095B131_SCHHA	
Ubiquitin	A0A0R5RJE0_SCHMA	

Table 2: Proteomic analysis of most abundant S. mansoni proteins identified in EVs

Protein content of *S. mansoni* cercarial EVs. Mass spectrometry data was searched in NCBI *S. mansoni* genome datasets. Proteins had at least two positives across triplicates of two independent experiments. Proteins that have been identified in proteomic analyses of *S. mansoni* cercariae and schistosomula excretory secretory products were indicated. Relevant references were provided (refer to reference list for full citations). Proteins in boldface print are on ExoCarta's "top 25" list of most commonly found exosome markers from different species and tissues. Proteins with an asterisk indicates a current *S. mansoni* vaccine candidate.

2.3 Discussion

Over 700 million people are at risk of contracting schistosomiasis [1]. This debilitating disease has no preventative vaccine and currently one widely used treatment, praziquantel [1]. Therefore, the lack of vaccines and therapeutics for schistosomiasis justifies our study of *Schistosoma* extracellular vesicles. Parasite-derived extracellular vesicles have been shown to impact disease progression and promote parasite survival through their transfer of exosomal contents to host cells [244, 269, 290, 291]. This has been demonstrated in *Leishmania*, *Trypanosoma*, and *Plasmodium* [244, 269, 290, 291]. The study *Schistosoma* EVs is a growing field as the evidence for their involvement in parasite-parasite and host-parasite communication

are being discovered [249]. Currently, most *Schistosoma* exosome and EV research has been focused on the adult and egg life cycle stages [250-257]. Therefore, the characterization and analysis of cercarial EVs especially those secreted during infection and transformation has not been studied yet. We demonstrate that *S. mansoni* release EVs during infection. We also characterized the proteomic profiles of the EVs.

We purified *S. mansoni* cercariae secreted EVs through a novel isolation procedure. This heat induced EV release protocol included 0.45 μm filtration and differential ultracentrifugation at 100,000 x g to ensure the isolation of small EVs. Through biophysical characterization such as transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) with Nanosight, and proteomics, we can suggest that the EVs collected are exosomes. The EVs collected were roughly 100-130 nm in diameter and many proteins identified were considered exosome markers such as HSP70, CD63, and enolase (Table 1 and 2, Figure 2) [263].

Although this protocol resulted in the isolation of exosome-like vesicles, it is important to analyze why other procedures attempted were not optimal. First, we experimented with different media types. From Tekwu *et al.*, they demonstrated that DMEM and RPMI 1640 alone resulted in slowed and less dynamic motility schistosomula post transformation [292]. Similarly, we observed decreased motility when the *S. mansoni* cercariae and schistosomula were incubated from 4 hours to 24 hours. This could be due to a prolonged incubation period without media supplementation as schistosomula reside within the host's dermis and have access to an abundance of nutrients. Therefore, we chose to add 5% exosome-depleted FBS and antibiotics to RPMI 1640 and DMEM because groups have shown that recently transformed schistosomula had increased viability compared to media without supplementation [293, 294]. When cercariae and schistosomula were cultured in supplemented media, the parasites were healthier as they were more motile. However, with TEM analysis, there were low concentrations of EVs and a high concentration of background proteins from the FBS (Appendix 4). Thus, we moved back to using RPMI 1640 without phenol red without supplementation as parasites were slightly healthier in this media opposed to DMEM. Next, we varied different incubation periods to combat the quick decline of cercariae and schistosomula health during incubation. Lastly, mechanical transformation with vigorous agitation resulted in many dead parasites. This could be due to parasite damage from mechanical transformation. Based on MicroBCA, NTA, and TEM results, there was a low concentration of EVs secreted by the parasites in 4-hour and overnight incubations with and without agitation. Since

each group contained roughly 3,000 cercariae each, the low concentration of EVs may be due to low number of parasites available. Moreover, low EV yield could be attributed to isolation technique. During these trials, the supernatant post-incubation was filtered with a 0.22 μm syringe and thus could have removed aggregates of EVs. It is also important to note that during cell apoptosis, apoptotic bodies are can be secreted by eukaryotic cells [295]. However, they were not isolated as apoptotic bodies range from 500-1000 nm in diameter and the final procedure involved 0.45 μm filtration.

Some of the proteins identified in *S. mansoni* EVs are involved in metabolic processes. These proteins are vital for parasite survival and disease progression. Serpin is a serine proteinase inhibitor that inhibits cercarial proteases through complex formation [282]. Typically, serpins, like serine serpin isoform 3 (SmSPI), modulate serine proteases in *Schistosoma* and their host to facilitate intradermal and intravenous survival [278]. Additionally, Pakchotanon *et al.* showed that cercariae had SmSPI levels 20 times less than schistosomula throughout their whole bodies [278]. Therefore, serpin being released in EVs during the time of infection may have a role in parasite maturation and modulating host serine proteases in the dermis to facilitate cercariae invasion. *S. mansoni* enolase (SmEno) is a host-interactive tegumental enzyme that can bind to and enhance human plasminogen activation to degrade blood clots to aid in parasite migration. Figueriedo *et al.* demonstrated that SmEno highly expressed in the schistosomula, adult, and egg stages [277]. However, when SmEno was blocked with RNA interference, plasminogen activity was unaffected [277]. Therefore, *S. mansoni* employs different enzymes to promote migration and prevent blood clotting [277]. Furthermore, measuring *S. japonicum* enolase levels in serum can be used to detect active *Schistosoma* infection [296]. This has not been analyzed with *S. mansoni* but with our findings and knowledge that SmEno is expressed in schistosomula, the detection of SmEno may be used as a detection method. Thus, SmEno derived from EVs may be involved in early parasite migration in the host circulation by inhibiting host plasminogen activity.

During infection, cercariae release their pre-acetabular glands and we demonstrated that EVs are also secreted. Therefore, investigating their proteomic profiles is important in understanding the potential role of EVs during parasite penetration. Paveley *et al.* visualized the uptake of excretory secretory molecules from cercariae by MHCII cells within two hours of infection [297]. As seen in table 2, we identified four overlapping proteins, 14-3-3 homolog 1, HSP70, cercarial protease, and elongation factor 1-α, that were also found during cercariae-

schistosomula mechanical transformation and skin-invasion *ex-vivo* [102, 265, 277]. Out of these proteins, HSP70, 14-3-3 proteins, and elongation factor 1-α are enriched in exosomes and are considered to be exosome markers [263, 264]. We can suggest that presence of these proteins in parasite secretions may be due to the presence of EVs. Therefore, most proteins that we identified were not found in cercariae and schistosomula excretory and secretory products. *S. mansoni* EVs may be internalized by host cells and these packaged proteins could potentially alter physiological and pathological states of their target cells through horizontal protein transfer. As shown in table 3, many of the proteins identified have important functions that allow for *Schistosoma* survival. Therefore, by packaging these important proteins into EVs, the *Schistosoma* proteins avoid immune detection and aid in parasite survival.

Protein Name	Important functions in *Schistosoma*	References
14-3-3 protein homolog 1	Protein synthesis, epithelial cell growth by Akt/mTOR pathway stimulation	256,257
Albumin	Neutralizes host oxidative immune response	261,262
Calmodulin	Calcium-mediated signaling that stimulates muscle contraction, calcium homeostasis, and inflammation	259
Cercarial protease	Breaks down host elastin to allow for parasite penetration	262, 263
cGMP-dependent protein kinase	Triggers serine/threonine kinase activity in nitric oxide and atrial natriuretic peptide signaling cascades	260
EF-hand domain-containing protein	Could assist schistosomes in escaping host immune attacks through inhibiting chemotaxis and non-complement fixing antibody responses	263
Enolase	Enhances plasminogen activation to degrade blood clots to aid in parasite migration	269
Serpin, putative	Inhibits cercarial protease function through complex formation	267

Table 3: Key Schistosoma proteins identified from S. mansoni cercarial EVs with functions

Protein content of *S. mansoni* cercarial EVs. Mass spectrometry data was searched in NCBI *S. mansoni* genome datasets. Important proteins in our analysis was determined by their roles in *Schistosoma*. Relevant references were provided (refer to reference list for full citations).

Next, we compared the protein contents of cercarial EVs from table 1 to the proteomic profiles of *S. mansoni* cercariae and schistosomula [298-300]. The common proteins between cercarial EVs and cercariae and schistosomula whole parasite proteomics are 14-3-3 homolog 1, actin, elongation factor 1-α, GAPDH, HSP70, serpin, enolase, and ubiquitin. However, the whole parasite proteomic results focused on the most abundant or soluble proteins and they did not publish all proteins found. Additionally, the groups differed in proteomic analysis techniques. Many of the proteins we identified in cercarial EVs were not found in published whole parasite proteomic data. We suggest that the cercarial EV derived proteins originated from *Schistosoma* because the proteins identified were identified as *Schistosoma* proteins.

We identified CD63 which is a homolog of *S. mansoni* tetraspanin Sm-TSP-2. It is a vaccine candidate able elicit a high level of serum Sm-TSP-2 specific IgG that is protective against *S. mansoni* infection in humans [274]. The Sm-TSP-2/Alhydrogel® vaccine is undergoing clinical trials [274]. Therefore, there may be potential in developing strong IgG immune response against

S. mansoni CD63 with repeated exposures to cercarial EVs. Furthermore, we were able to identify *Schistosoma* vaccine candidate Sm29 [150]. Mice immunized with recombinant Sm29 had a 51% reduction in adult worm burden and 50% reduction in liver granuloma counts [150]. Cardoso *et al.* demonstrated that high Sm29 IgG1 and IgG3 levels corresponded to individuals with natural resistance to infection and individuals resistant to re-infection post-PZQ treatment [195]. This may imply that repeated exposure to Sm29 through *S. mansoni* cercarial EVs can potentially play a role in immunity against cercarial infection. This is also known as concomitant immunity.

Many proteins identified in our samples were involved in signal transduction and biological regulation such as 14-3-3 *S. mansoni* homolog and calmodulin. Dovrat *et al.* demonstrated that exosome-derived 14-3-3 induces Wnt, meaning Wingless and Int-1, signaling activity in host cells [301]. Wnt signaling is critical for development, differentiation, and cellular homeostasis. It has also been shown to be associated with exacerbated liver fibrosis when added exogenous to *S. japonicum* infection [302]. Therefore, we are suggesting that *S. mansoni* cercariae EVs may be involved in modulating host Wnt signaling. Furthermore, calmodulin has been shown to be involved in vesicular trafficking and exosome secretion, so it may be involved in cercarial exosome release at the time of infection [303].

Winkel *et al.* showed that excretory secretory molecules released by cercariae enhanced the production of IL-10, IL-6, and macrophage inflammatory protein-1α in the dermis after three hours of exposure [110]. With further analysis, they found that DDCs had an increased expression of programmed death ligand 1 and 2 (PDL-1/2), and IL-10 production [110]. Thus, cercarial excretory secretory molecules induce a regulatory immune response which may aid in parasite survival [110]. It can be hypothesized that cercarial EVs may stimulate a similar response when exposed to DDCs and also have an immunoregulatory effect on the dermis. However, further studies involving *ex vivo S. mansoni* infections and EV-primed mouse infection models would be interesting to pursue to understand the complex relationship between *S. mansoni* EVs and the human host.

Protein concentrations were lower than expected for proteomic analysis due to loss of protein during precipitation. One of the steps of TCA protein precipitation involved aspirating supernatant to leave a protein pellet. Thus, we hypothesize that some of the pellet was removed during this step and this resulting in lower protein concentrations. Additionally, another reason for low protein concentrations may be due to improper MicroBCA preparation. For S2, the protein

concentration from the MicroBCA analysis was 504.82 μg/mL. For the TCA preparation, we isolated 5 μg based on our MicroBCA analysis. Thus, if our initial protein concentration was over exaggerated due to external proteins, we may have isolated too little for our proteomic analysis. Therefore, proteins that had single hits were also analyzed as they belonged to *Schistosoma*. We hypothesize that with adequate protein concentrations, these proteins would have more peptides identified. Some interesting proteins identified were ATPDase-1 and GAPDH. ATPDase-1 is a membrane-associated protein that inhibits platelet aggregation through the hydrolysis of extracellular prothrombotic ATP and ADP [289]. GAPDH is found on the surface of the parasite and is involved in blood clot degradation [300, 304]. Pirovich *et al.* developed a recombinant GAPDH which was shown to binds to and activates plasminogen which can degrade blood clots around the parasite [304]. Since both proteins are involved in the degradation of blood clots, cercarial EVs may facilitate parasite migration through the host.

The goal of our project was to not only hypothesize potential roles of cercarial EVs but to also identify hopeful vaccine or treatment candidates for *S. mansoni*. SmEno would be an interesting treatment target as its inhibition may lead to parasite death because of its involvement in parasite migration. SmEno is a 75% homolog to α-enolase isoform 1 in humans using the compositional score matric adjustment method [305]. Therefore, designing a recombinant protein that is unique to *S. mansoni* for vaccine usage is essential to prevent cross-reactivity with the human homolog.

Other vaccine targets may be cercarial proteases. Many groups have tested the efficacy of protease inhibitors as a way to prevent different parasitic infections such as *Cryptosporidium parvum* and *Trypanosoma cruzi* [306-309]. Ndao *et al.* demonstrated that the vinyl sulfone inhibitor K11777 rescues C57BLL/6 IFN-γ knockout mice from lethal *C. parvum* infection when it was administered orally or intraperitoneally [307]. This is interesting as their mouse model mimicked an AIDs patient's immune system and thus is highly susceptible to *C. parvum* infections [307]. For *T. cruzi,* research groups have shown that K11777 prevented cardiomyopathy after 7 days of treatment in a dog model of *T. cruzi* infection [306]. Also, K11777 rescued mice with lethal doses of *T. cruzi* after 27 days of treatment [308]. Some groups have demonstrated that serine protease inhibitors can prevent *Schistosoma* infection. For example, Lim *et al.* formulated a mixture of different irreversible serine protease inhibitors in a propylene glycol and isopropyl alcohol with Topicare Delivery Compounds® [310]. The topical cream was applied on mice skin

prior to infection [310]. It reduced *S. mansoni* worm burden and egg burden 80% and 92%, respectively [310]. K11777 has also been used to treat *S. mansoni* infection via intraperitoneal administration twice a day during schistosomula migration (1- 14 days post infection) and during egg-laying stage of adult worms (30 – 37 days post infection) in BALB/c mice [311]. When K11777 was administered during larval migration, it eliminated all parasite eggs [311]. And when administered during egg-laying, K11777 reduced egg burden by 81% [311]. We propose that an intradermal vaccine targeting cercarial proteases may limit and even prevent cercarial infection. An intradermal vaccine would be interesting to develop as it is where the parasite invades the human host. Dupré *et al*. demonstrated that intradermal vaccination of *S. mansoni* 28 kDa glutathione S-transferase (Sm28GST) induced long-lasting specific IgG antibody response in sera of immunized rate [312]. Based on their work, an intradermal vaccine is a viable option for delivery. Therefore, developing an intradermal vaccine against secreted cercarial proteases may elicit long term protection against invading *S. mansoni* cercariae.

Furthermore, our *in vitro* EV isolation procedure may not reflect a natural infection. A potential future direction for this project is to design different infection models such as human skin *ex vivo* experiments to allow for the isolation of *S. mansoni* EVs. It would also be interesting to analyze horizontal transfer of *S. mansoni* EV-derived proteins to host cells. Mondal *et al.* created a procedure that allowed for visualizing EVs using immune-fluorescence based technique [313]. They tagged glioblastoma cell secreted EVs with antibodies that are commonly found on EVs like CD63 and HSP70 [313]. This group used this procedure to analyze the localization of EVs after its uptake by recipient cells [313]. We could potentially use this procedure to not only analyze *S. mansoni* EV uptake by dermal DCs *in vitro,* but we can also inject skin explants with tagged EVs and analyze its localization during infection. The effect of cercariae EVs can also be studied via priming mice with cercariae EVs prior to *S. mansoni* infection. Furthermore, our project focused on exosome-like EVs and studying the protein contents of other EVs secreted by *S. mansoni* cercariae during transformation such as microvesicles (50- 1,000 nm) [314]. Microvesicles biogenesis involves the outward blebbing and pinching of the plasma membrane and releasing the resulting microvesicles into the extracellular space [314]. They have been shown to carry host cell nucleic acids, lipid, and proteins [315]. For example, microvesicles derived from human bone marrow mesenchymal stem cells (MSCs) have been shown to inhibit cell growth of tumor cell lines such as HelpG2, Skov-3, and Kaposi cells [316]. These tumor cells were injected into SCID

mice, genetic immune deficient mice, to generate establish tumors. *In vivo* intra-tumor administration of MSC microvesicles significantly inhibited tumor growth [316].

In conclusion, we developed a novel technique to isolate *S. mansoni* EVs secreted when cercariae transform into schistosomula. This method resulted in a high yield of homologous exosome-like EVs. Our proteomic analysis demonstrated that many of the EV derived proteins were not found in cercariae or schistosomula secretions. Therefore, *S. mansoni* may produce EVs to protect vital proteins for parasite survival from host immune detection. Additionally, EVs contain proteins that are necessary for vesicle structure and may not have a role in disease progression. Thus, through proteomic analysis we are furthering our understanding on the role of *S. mansoni* EVs during infection. This insight may lead to the development of novel therapeutics and vaccines effective against *Schistosoma*.

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Appendix

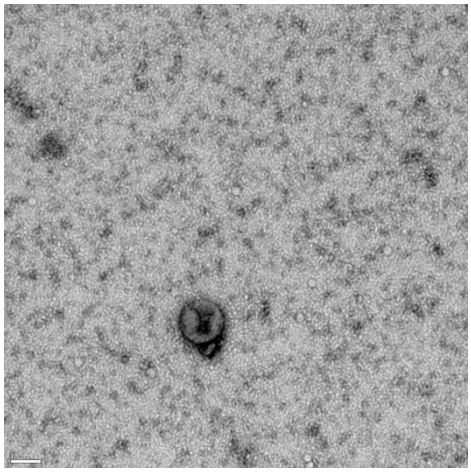
Supplemental Documents

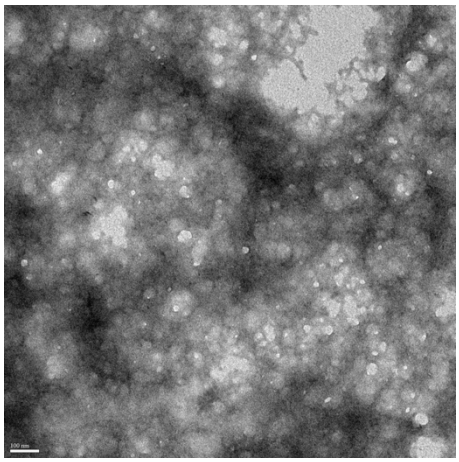
A1: Total Unique Peptide of *Schistosoma mansoni* cercariae EVs (Proteomic Analysis)
smev.cc/A1

A2: *Schistosoma mansoni* cercariae EV derived proteins with single positives
smev.cc/A2

A3: List of uncharacterized *Schistosoma mansoni* proteins from cercariae EVs
smev.cc/A3

A4: TEM images of EV isolation with DMEM and RPMI 1640 without phenol red supplemented with 5% exosome depleted FBS and 200 ug/mL streptomycin and penicillin





A5.4: Transmission electron microscopy photos of S. mansoni cercariae EVs in DMEM and RPMI 1640 without phenol red (supplemented with 5% exosome depleted FBS and 200 ug/mL streptomycin and penicillin)

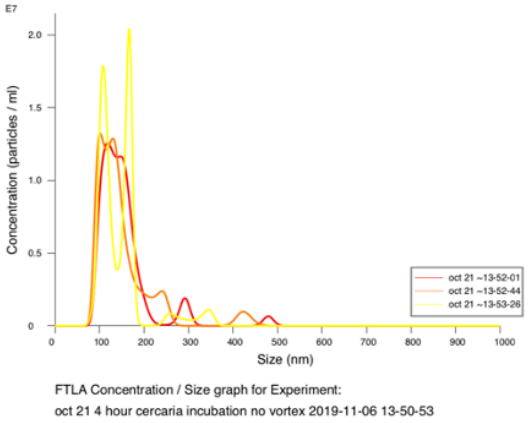
The analysis was performed from EVs suspension extracted from secretions from *S. mansoni* cercariae during *in vivo* heat shock in DMEM (Right) and RPMI 1640 (Left) supplemented supplemented with 5% exosome depleted FBS and 200 ug/mL streptomycin and penicillin to stimulate transformation into schistosomula. The EVs were stained with uranyl acetate to visualize morphology. Images were taken at 32000x; scale bar represents 100 nm.

A5: MicroBCA, Nanosight, and TEM figures for *S. mansoni* cercarial EVs collected by agitation and heat induced cercariae transformation into schistosomula

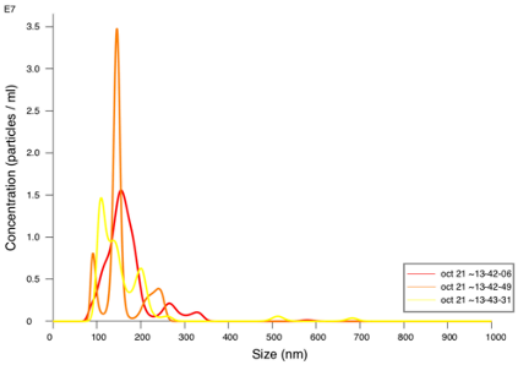
	4-hour incubation	4-hour incubation with agitation	ON incubation	ON incubation with agitation
Protein Concentration (µg/mL)	-2.42287	-3.05482	-2.94833	-0.66361

A5.1: MicroBCA of S. mansoni cercarial EVs collected by agitation and/or heat induced cercariae transformation into schistosomula

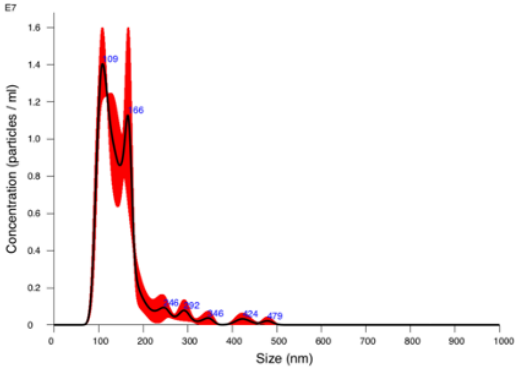
MicroBCA results are averages of two separate preparations. The protein concentration was calculated from a 1:10 dilution of the samples used in the assay. ON indicates overnight.



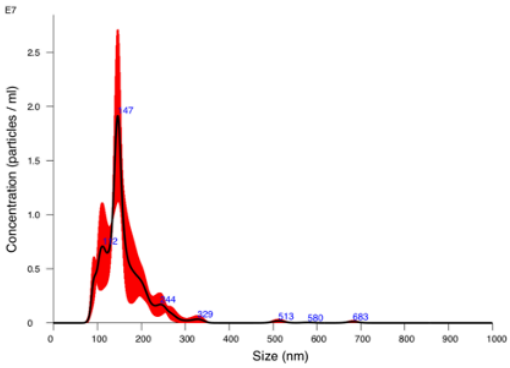
FTLA Concentration / Size graph for Experiment:
oct 21 4 hour cercaria incubation no vortex 2019-11-06 13-50-53



FTLA Concentration / Size graph for Experiment:
oct 21 4 hour cercaria incubation 2019-11-06 13-40-28



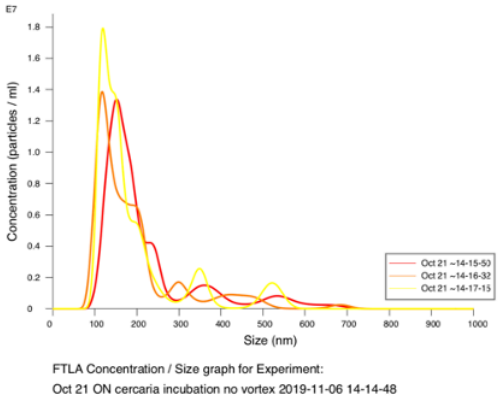
Averaged FTLA Concentration / Size for Experiment:
oct 21 4 hour cercaria incubation no vortex 2019-11-06 13-50-53
Error bars indicate + / -1 standard error of the mean

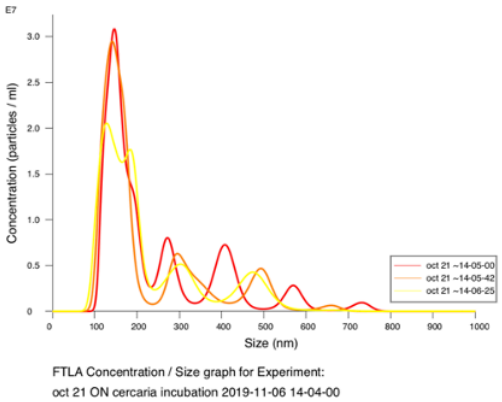


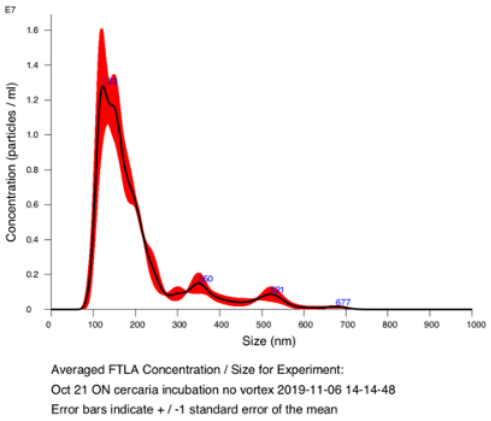
Averaged FTLA Concentration / Size for Experiment:
oct 21 4 hour cercaria incubation 2019-11-06 13-40-28
Error bars indicate + / -1 standard error of the mean

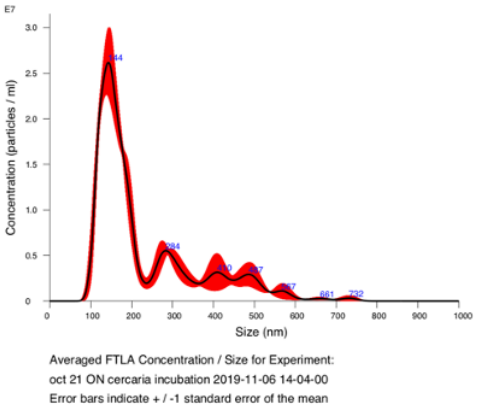
A5.2: Nanoparticle tracking analysis of S. mansoni cercariae EVs collected by agitation and/or heat induced cercariae transformation into schistosomula with a 4-hour incubation

The analysis was performed from EVs suspension extracted from secretions from *S. mansoni* cercariae during in vivo heat shock and agitation to stimulate transformation into schistosomula. Representative Nanosight generated graphs of nanoparticle size distribution and concentration in particles per mL. On the top, each line represents the concentration in one video. On the bottom are the average concentrations calculated from the three videos taken. Graphs are from a single preparation. On the left: 4-hour incubation without agitation. On the right: 4-hour incubation with agitation.



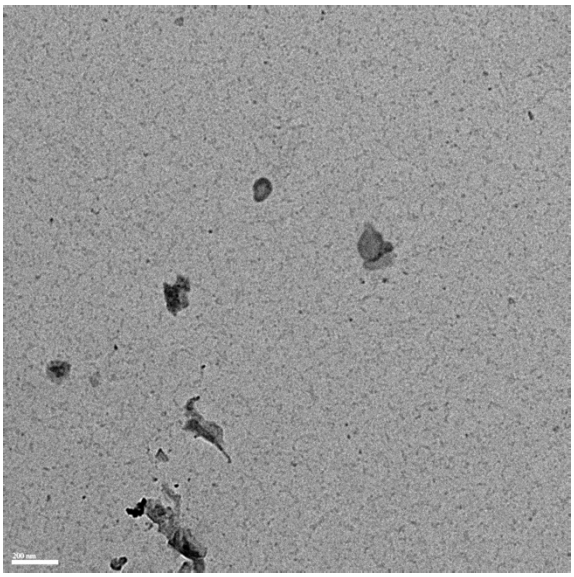






A5.3: Nanoparticle tracking analysis of S. mansoni cercariae EVs collected by agitation and/or heat induced cercariae transformation into schistosomula with a 4-hour incubation

The analysis was performed from EVs suspension extracted from secretions from *S. mansoni* cercariae during in vivo heat shock and agitation to stimulate transformation into schistosomula. Representative Nanosight generated graphs of nanoparticle size distribution and concentration in particles per mL. On the top, each line represents the concentration in one video. On the bottom are the average concentrations calculated from the three videos taken. Graphs are from a single preparation. On the left: overnight incubation without agitation. On the right: overnight incubation with agitation.



A5.4: Transmission electron microscopy photos of S. mansoni cercariae EVs collected by agitation and/or heat induced cercariae transformation into schistosomula

The analysis was performed from EVs suspension extracted from secretions from *S. mansoni* cercariae during *in vivo* heat shock without agitation RPMI 1640 to stimulate transformation into schistosomula. This sample was obtained from parasites that were incubated for 4-hours. The EVs were stained with uranyl acetate to visualize morphology. Images were taken at 32000x; scale bar represents 100 nm.