Identification of candidate serum biomarkers for schistosomiasis infection using mass spectrometric approaches

By

Manal Ibrahim Kardoush

Institute of Parasitology

McGill University, Montreal

March, 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Parasitology

CONTENTS

LIST OF ABBREVIATIONS

°C	Degrees Celsius
1-D	One-dimensional
2-D	Two-dimensional
2DE	Two-dimensional gel electrophoresis
A1AT	α 1-antitrypsin
ACN	Acetonitrile
ADCC	Antibody-dependent cell mediated cytotoxicity
ApoA1	Apolipoprotein A-I
BP	Before the present
B	*Biomphalaria*
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CHAPS	Cholamidopropyl dimethylammonio-1-propanesulfonate
CHCA	Cyanohydroxycinnaminic acid
CID	Collision induced dissociation
CM10	Cation exchange
COPT	Circumoval precipitin test
Da	Dalton(s)
DC	Dendritic cells
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FWHM	Full width at half maximum
g	Gram(s)
GGT	γ-glutamyl transferase
h	Hour(s)
HCl	Hydrochloric acid
HDL	High density lipoprotein
HCD	Higher energy collision dissociation
HIV	Human immunodeficiency virus
ICAT	Isotope-coded affinity tag
Ig	Immunoglobulin
IFN	Interferon
IMAC	Immobilized metal affinity capture
IHAT	Indirect hemagglutination test
IFT	Indirect immunofluorescence test
IL	Interleukin, molecules produced by immune system cells
kDa	Kilodaltons(s)
kV	Kilovolt
L	Liter(s)
LC	Liquid chromatography
LC-MS	Liquid chromatrography coupled with mass spectrometry
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry

LTQ	Linear trap quadrupole
m/z,	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
min	Minute(s)
mol	Mole(s)
MRM	Multiple reaction monitoring
MW	Molecular weight
NCBI	National Center for Biotechnology Information
NK	Natural killer
OD	Optical density
P value,	Probability value
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline + Tween 20
PCR	Polymerase chain reaction
PES	Polyethersulfone
pI	Isoelectric point
PAI-2	Plasminogen activator inhibitor-2
PTMs	Post-translational modifications
PQD	Pulsed-Q-dissociation
PR	Putative resistant
RP	Reverse-phase

RNA	Ribonucleic acid
S	*Schistosoma*
s	Second(s)
SA	Sinapinic acid
SRM-MS	Selected reaction monitoring-mass spectrometry
SARS	Acute Respiratory Syndrome
SDS-PAGE SDS	Polyacrylamide gel electrophoresis
SELDI	Surface-enhanced laser desorption/ionization
SILAC	Stable isotope labeling amino acids cell culture
SWAP	Soluble worm antigen preparation
TFA	Trifluoroacetic acid
Th1	T helper cell type 1
Th2	T helper cell type 2
TOF	Time-of-flight
SmTSP-2	*Schistosoma mansoni* tetraspanin
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Schistosome infections, caused by a family of helminth parasites, are major neglected tropical diseases (NTDs) that have serious implications for socio-economic development in many tropical countries. These debilitating and chronic diseases are endemic in more than 70 countries but diagnostic tools are limited and new tests are necessary to limit morbidity and mortality. The development of such tests may be facilitated by the identification of disease-specific biomarkers that are positive early in infection, that can distinguish between acute and chronic infection and that can serve as validation of cure. This thesis work has two major goals: (a) to identify stage-specific proteomic patterns during early (3 week), acute (6 week) and chronic (12 week) *Schistosoma mansoni* infection in a mouse model; (b) to profile the sera of humans infected by *S. mansoni*, or *S. haematobium* and compare these patterns with the protein profile of healthy human serum. To achieve these goals, proteomic analyses were performed on mouse and human sera using several different mass spectrometry (MS) methodologies. These investigations not only identified large numbers of host proteins/protein peaks that are up- or down-regulated in infected sera (mouse and human) but also found large numbers of schistosome-origin proteins in the serum of infected mice. The presence of host proteins, such as transferrin and alpha 1-antitrypsin, as well as one of the schistosome proteins, glutathione S-transferase (GST), were confirmed by Western blot. In addition to these disease- and stage-specific protein profiles, we also identified a number of host proteins that may individually have potential as novel diagnostic tests for schistosome infections in humans, including apolipoprotein A-I (Apo A-I: up-regulated in chronic *S. haematobium* infected patients) and carbonic anhydrase 1 (CA1: down-regulated in chronic *S. mansoni* infected patients). If confirmed in larger field studies, these novel

biomarkers, and others yet to be identified from our large MS databases, have the potential to contribute significantly to schistosomiasis detection and subsequent eradication efforts.

RÉSUMÉ DE THÈSE

La bilharziose ou schistosomiase, infection parasitique, demeure l'une des plus importantes maladies tropicales qui sont toujours négligées. Ces maladies débilitantes et chroniques ont des conséquences sérieuses pour le développement socio-économique des pays tropicaux. Elles sont endémiques dans plus de 70 pays, mais il est toujours nécessaire de trouver de nouveaux instruments d'intervention pour réaliser un diagnostic précis des cas d'infection afin d'empêcher la morbidité et la mortalité. Il est peut-être possible de réaliser de nouveaux tests diagnostiques en identifiant des biomarqueurs spécifiques qui deviennent positifs au début de l'infection. L'identification de protéines associées aux différentes maladies pourrait indiquer de nouvelles formes de diagnostiques, qui peuvent être utilisées pour le diagnostic des phases aiguës et chroniques de l'infection et pour la validation du traitement. Cette étude a deux objectifs: (a) identifier des modèles protéomiques dans les sérums pour les différentes phases de l'infection, en commençant par la phase préliminaire (3 semaines), puis en étudiant la phase aiguë (6 semaines) et ensuite la phase chronique (12 semaines) des infections à *Schistosoma mansoni* dans un modèle de souris; (b) établir le profil des sérums d'humains infectés par *S.mansoni* et *S. haematobium* et le comparer avec un profil de sérums sains. En vue de cela, des analyses protéomiques ont été effectuées sur les sérums d'humains et de souris en utilisant de puissantes méthodes à haut débit. Ces recherches ont non seulement identifié des protéines de l'hôte/des quantités maximums de protéines qui sont régulés en hausse et en baisse dans les sérums infectés (d'humains et de souris), mais ont aussi caractérisé les protéines de schistosome dans les sérums de souris infectées.

La présence de protéines de l'hôte, telles que la transferrine et l'alpha 1-antitrypsine, ainsi qu'une des protéines de schistosome, la glutathion S-transférase (GST), a été confirmé par

le Western Blot. Les résultats ont montré que les profils de protéines sont liés aux différentes maladies et aux phases de l'infection. Ils ont aussi identifié certains protéines de l'hôte qui peuvent servir individuellement comme de nouveaux tests diagnostiques pour la schistosomiase, comme l'apolipoproteine A-I (Apo A-1 : régulée en hausse auprès des patients chroniques qui sont infectés par *S. haematobium*) et l'anhydrase carbonique 1 (CA1 : régulée en baisse auprès des patients chroniques qui sont infectés par *S. mansoni*). Si des études plus vastes confirment ces résultats, ces nouveaux biomarqueurs, et aussi d'autre biomarqueurs qui n'ont pas encore été identifiés à partir de nos grandes banques de données MS, pourraient contribuer de manière importante à l'éradication de la schistosomiase.

ACKNOWLEDGEMENTS

In the name of *Allah* (God), the most Gracious and the most Merciful, who best owed upon me enough guidance and benevolence to carry out this work.

I would like to thank all the people who have helped me during the time I have spent in the laboratory and during the writing of this thesis. I am very grateful to my supervisors, Dr. Brian Ward who has always been a very encouraging supervisor, and definitely a distinguishable scientist. Thanks for teaching, supporting, and guiding me throughout my PhD journey. I would like to extend my deepest gratitude to Dr. Momar Ndao, who supported me throughout my research. This thesis would not have been possible without their guidance and support.

I like to acknowledge the members of my PhD advisory and oral examination committee, Dr. Paula Ribeiro and Dr. Timothy Geary for their valuable suggestions.

I want to extend my thanks to the Institute of Parasitology at McGill University, as well as the staff I had the honour and privilege of working with at the National Reference Centre for Parasitology (NRCP) at the Montreal General Hospital.

In addition, I gratefully acknowledge the Egyptian Government that sent me abroad for studying and increasing my knowledge, and for funding me throughout this research.

I would also like to thank all my friends and family for their support and interest in my research, in particular my parents, and my sisters. I am ever grateful to my husband, Ahmed for putting up with me at my worst and encouraging me to finish despite all his responsibilities and commitments, and my children for their patience and support.

PREFACE

The focus of this thesis is to establish a diagnostic test for schistosomiasis with the ultimate goal of studying the proteomic profiles of sera from control and infected mice and humans in order to identify the possible diagnostic biomarkers. The project was undertaken as follows:

1. Study of protein profiles in a mouse model of schistosomiasis using different mass spectrometry platforms yielded several biomarkers that might be helpful in the diagnosis of schistosomiasis.

This phase of the study is accepted for publication in Plos One as: Kardoush MI, Ward BJ, Ndao M. Identification of candidate serum biomarkers for *S. mansoni* infected mice using multiple proteomic platforms (In Preparation).

2. Using the same mass spectrometry techniques in mouse and human samples, we were able to identify CA1 as a novel biomarker for schistosomiasis in humans and animal models.

The second phase of the study is accepted with minor revision for publication in American Journal of Tropical Medicine & Hygiene - AJTMH as: Kardoush MI, Ward BJ, Ndao M. Carbonic anhydrase 1 is a novel biomarker for diagnosis of human Schistosoma mansoni infection.

3. Comparison of protein profiles in human infected with *S. haematobium*, and control samples resulted in valuable diagnostic biomarkers that differentiate the healthy from the infected patients.

The third phase of the study is in preparation for publication as: Kardoush MI, Thiam A, Faye B, Gaye O, Ndao M, Ward BJ. Mass spectrometric approaches for the identification of candidate serum biomarkers for *Schistosoma haematobium* infection (In Preparation).

CONTRIBUTION OF AUTHORS

Manuscript 1:

Identification of Candidate Serum Biomarkers for *Schistosoma mansoni* Infected Mice Using Multiple Proteomic Platforms

Design of the study: Brian J. Ward, Momar Ndao & Manal I. Kardoush

Execution of experiments: Manal I. Kardoush

Analysis of data: Manal I. Kardoush, Brian J. Ward, & Momar Ndao

Writing of the manuscript: Manal I. Kardoush, Brian J. Ward, & Momar Ndao

Manuscript 2:

Carbonic anhydrase 1 is a novel biomarker for diagnosis of human *Schistosoma mansoni* infection

Design of the study: Brian J. Ward, Momar Ndao & Manal I. Kardoush

Execution of experiments: Manal I. Kardoush

Analysis of data: Manal I. Kardoush, Brian J. Ward, & Momar Ndao

Writing of the manuscript: Manal I. Kardoush, Brian J. Ward, & Momar Ndao

Manuscript 3:

Mass spectrometric approaches for the identification of candidate serum biomarkers for *Schistosoma haematobium* infection

Design of the study: Momar Ndao, Brian J. Ward & Manal I. Kardoush

Execution of experiments: Manal I. Kardoush, A. Thiam, B. Faye, O. Gaye

Analysis of data: Manal I. Kardoush, Brian J. Ward, & Momar Ndao

Writing of the manuscript: Manal I. Kardoush, Brian J. Ward, & Momar Ndao

CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Schistosomiasis

Human schistosomiasis, also call bilharzia, was first identified by Theodor Bilharz, a German pathologist working in Egypt in 1851. Historically however, human schistosomiasis was occurring a long time before this date. Several studies have shown the presence of schistosome parasites in Egyptian mummies (circa 3000 BC to 550 AD: Cockburn et al., 1975; Reyman et al., 1977; Lewin, 1977; Contis & David, 1996). Others have shown *Schistosoma japonicum* in a mummified body of the Han Dynasty (circa 2100 BP: Wei, 1973).

Parasitic blood flukes of the Trematoda class (phylum Platyhelminthes) are the cause of schistosomiasis (Gryseels et al; 2006). Adult worms are 7–20 mm in length; their color is white or grayish, and they have a cylindrical body with ventral and oral suckers, a complex tegument, a blind digestive tract, and reproductive organs. Schistosomes are sexually dimorphic. The male has a gynaecophoric canal, in which it carries the longer and thinner female. Schistosomes ingest blood and globulins into a blind-ending digestive system and primarily use anaerobic glycolysis for energy production. Waste products of schistosome digestion/metabolism are regurgitated into the host's circulation (Gryseels et al; 2006). The adult female can ingest up to 33,000 red cells per hour as well as significant volumes of plasma rich in host proteins during development and egg production (Lawrence, 1973). The schistosome gut is lined by a syncytial epithelium called the gastrodermis, which is a major interface between the parasite and host. Gastrodermal cells have the ultrastructural appearance of secretory cells with an endoplasmic reticulum, Golgi apparatus, and numerous vesicles (Bogitsh, 1975). The lumen of the gut contains plasma

proteins, ingested host cells and parasite secretory products (Bogitsh, 1982). The tegument is the main external interface between the schistosome and the host. It is a lipid bilayer membrane which forms a syncytium. This tegument has an unusual structure created by surface cells that 'project' themselves through muscular layers to form an external surface with the plasma membrane at the bottom covered by the membranocalyx (Morris & Threadgold, 1968; Wilson & Barnes, 1974).

Human schistosomiasis is a major public health threat in many parts of the world. Human disease can be caused by six species of schistosomes, the most important of which are *S. mansoni* and *S. japonicum* causing primarily intestinal schistosomiasis, and *S. haematobium*, causing primarily urinary schistosomiasis. Much less common are *S. intercalatum, S. guineensis,* and *S. mekongi* all of which target intestinal tissues preferentially (Davis, 2009). In all species, infection occurs primarily by direct contact with fresh water harbouring cercariae (see **Life Cycle** below). Schistosomiasis is among the most important parasitic diseases with a significant socio-economic impact mainly in the tropics and subtropics (King et al., 2005). Though seldom fatal, schistosomiasis is a life-long disease with significant chronic morbidity due to repeated exposures in endemic regions (Enk et al., 2010; Patz et al., 2000; Ross et al., 2002). Much of the negative health impact of schistosomiasis is attributable to a lack of effective tools for diagnosis (Grenfell et al., 2012; Utzinger et al., 2011). Detection of the eggs in stool or urine is the gold standard method for the diagnosis of schistosomiasis, and the Kato-Katz technique is the most widely used microscopic method in epidemiological surveys that focus on *S. mansoni*. (Katz et al., 1972). However, a large number of cases remains undiagnosed by this method because of the limited amount of feces examined per slide (~42 mg) as well as low egg production by many chronically-infected patients in endemic areas (Katz et al., 1970). Immunodiagnostic methods

using a variety of schistosome antigens such as extracts of adult worms, cercarial antigens, or egg extracts are popular but often cannot differentiate schistosomiasis from other parasitic infections. In addition, these methods cannot be used to diagnose early stages of schistosomiasis since they typically become positive only 6 to 12 weeks after exposure (Jones et al., 1992; Sandoval et al., 2006). Furthermore, these assays cannot distinguish between on-going and past infection. At present, there are no reliable biomarkers that can be used in the diagnosis of schistosomiasis.

Mass spectrometry (MS) is rapidly gaining traction as a research tool (Petricoin, et al., 2002) to detect biomarkers in parasitic diseases such as Chagas diseases (Ndao et al., 2010) and *Entamoeba histolytica* (Makioka et al., 2007), as well as in cancer research (Villanueva et al., 2006; Guo et al., 2010; Karpova et al., 2010; Pusch & Kostrzewa 2005; Cox et al., 2015; Bhatt et al., 2015; Hadi & Jamal, 2015; Hong et al., 2015).

Life Cycle:

The life cycle of schistosomes is complex, including an asexual phase in the intermediate host (amphibious snails: *S. mansoni* infects *Biomphalaria*, *S. japonicum* infects *Oncomelania*, and *S. haematobium* infects *Bulinus* species) and sexual reproduction in a vertebrate host. Infections with human *Schistosoma* are caused by direct skin penetration upon contact with fresh water contaminated with cercariae. Free-living, non-feeding, cercariae escape through the birth pores of daughter sporocysts and emerge from the infected snail. Cercaria (total length ~325 μm) have a complex structure with two main parts: the body and the tail. The tegument is covered in a single outer membrane that coated in a thick glycocalyx (Hockley, 1972) that enables the cercaria to be water-impermeable (Stirewalt, 1963). The cercaria has two layers of muscle underlying the tegument; the outermost layer is circular, which encloses the inner longitudinal

layer (Mair, 2009). The acetabulum (ventral sucker) is situated two thirds of the way down the body. Ten large unicellular acetabular glands occupy 50-66% of the body mass; two pairs of glands lie anterior, and three pairs lie posterior to the acetabulum (Dorsey, 1975). The head is located at the anterior section of the body, and the mouth lies on the ventral side of the head. The mouth opens into the oesophagus, which runs through the posterior of the head, continues ventral to the brain (Ebrahimzadeh, & Kraft, 1969), to the blind bifid caecum. The gut luminal surface bears many plates. The ultrastructure of the cercarial nervous system has been described (Cousin, & Dorsey, 1991). The cercaria has a bilobed brain, which lies between, the head capsule, the pre-acetabular gland, and sixteen nerve trunks that exit the brain (Cousin, & Dorsey, 1991).

The cercariae penetrate human skin using proteolytic enzymes that facilitate their entry. During this process, the cercariae lose their tails, release the pre-acetabular gland contents (Dorsey, 1976; Crabtree & Wilson, 1985), lose their glycocalyx (McLaren, & Hockley, 1976) and form a new double outer membrane (Skelly, et al., 1994). By the second day, the schistosomulae become elongated, and there are fewer spines between the mouth and the ventral sucker (Crabtree, & Wilson, 1980). By the third day, all of the spines at the middle of schistosomulum have disappeared, but spines remain at the anterior and posterior ends (Crabtree, & Wilson, 1980). The maturing schistosomulum migrates to the lungs within 3-4 days, passing through the pulmonary capillaries and then entering the systemic circulation. During migration, the parasite undergoes further transformation and arrives in terminal venules at different sites according to the species: *S. mansoni* and *S. japonicum* are usually located in the superior mesenteric veins while *S. haematobium* targets the venous plexus of the urinary bladder. Upon

arrival in these target venules, the schsitosomulae mature into adult worms and initiate sexual reproduction with the production of eggs in an immature form.

Schistosome eggs consist of an egg shell of cross-linked proteins containing an ovum and vitelline cells. During the first 5–6 days in the host, the eggs mature with the formation of a miracidium. Each egg also produces a Von Lichtenberg's envelope from which proteins are secreted into the environment through pores (Neill et al., 1988; Ashton et al., 2001). Roughly half of the eggs produced succeed in exiting the host body by 'sticking' to the venule endothelium and migrating through the tissues to reach the intestinal (*S. mansoni* and *S. japonicum*) or bladder lumen (*S. haematobium*). The eggs achieve this migration by inducing granulomatous inflammation that breaks down the endo- and epithelial tissues and facilitates passage of the eggs into the faeces or urine respectively, leading to their excretion (Scheld, 2014). The other 50% of the eggs are retained in the tissues including those swept up the portal vein into the liver, where they induce the most serious pathological complications of disease (eg: fibrosis leading to cirrhosis)(Warren, 1978). When the eggs are released into freshwater, they hatch, releasing the miracidiae that infect the intermediate host (snail) to complete the Schistosome life-cycle. The miracidia do not feed and they die if they do not find an appropriate snail host within a few hours. Upon infection, the miracidium penetrates the head-foot of the snail and transforms into a mother sporocyst. The germ cells of the mother sporocyst then start dividing to produce daughter sporocysts. These daughter sporocysts migrate from the site of penetration to the digestive gland and reproductive tract of the snail. Eventually, germ cells within the daughter sporocysts begin to divide again, producing cercariae. Cercariae leave the snail at a high rate; up to several thousand per day and snails can continue to shed cercariae for months (Gryseels et al., 2006).

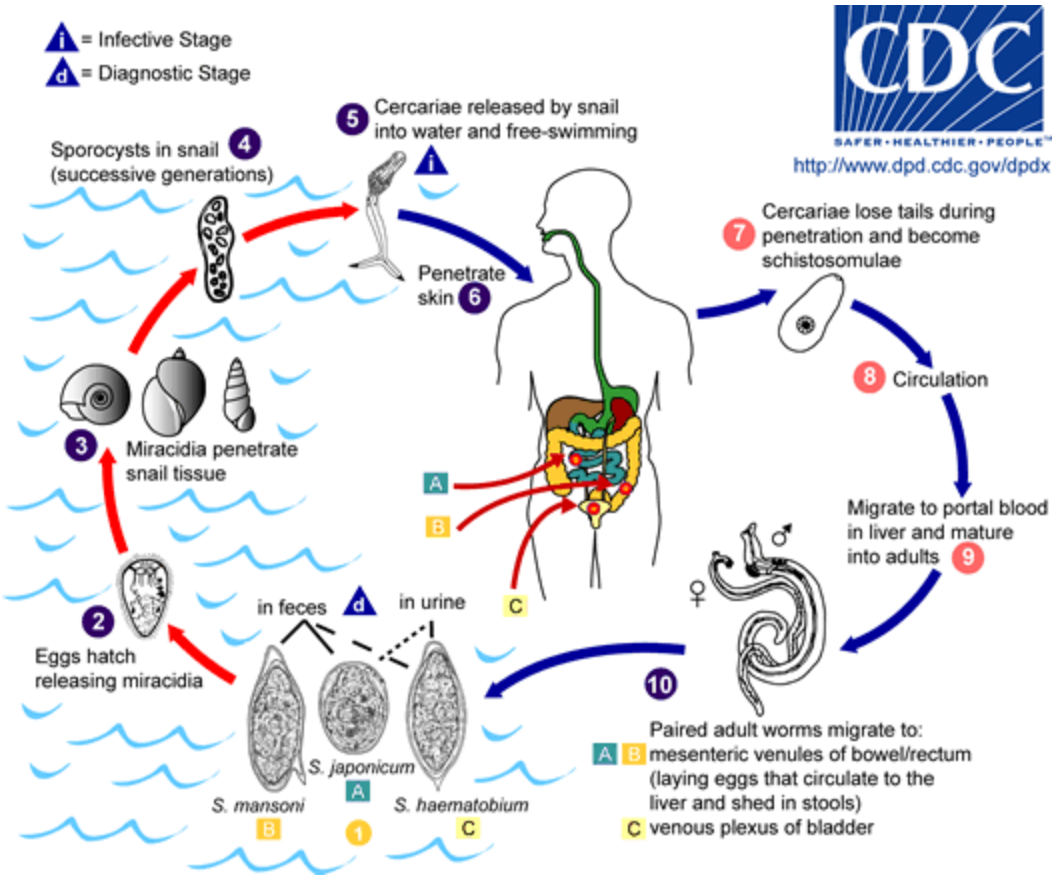

Figure 1. *Schistosoma* life cycle (Schistosomiasis. Centers for Disease Control, 2009).

Epidemiology:

Schistosomiasis is a tropical disease that has major public health impact in endemic countries. *Schistosoma mansoni* is distributed throughout Africa and South America while *S. haematobium* is distributed through Africa and in areas of the Middle East. *S. japonicum* is found in Indonesia and parts of China and Southeast Asia. *S. mekongi* occurs in the central Mekong Basin in Laos and Cambodia, and *S. intercalatum* is found in parts of Central and West Africa (Savioli et al., 2015). The various Schistosome species currently infect ~ 200 million people in over 70 countries of the world causing more than 200,000 deaths per year. More than 600 million people are at risk of acquiring one or more of these parasites (Bergquist, 2002; Engls, 2002). Teenagers have the greatest prevalence with generally higher rates in males compared to females

(Gryseels et al; 2006). According to WHO estimates (Gryseels et al., 2006), the total number of disability-adjusted life years (DALY) lost from schistosomiasis is ~1.532 million per year. At the current time ~77% of these DALYs lost are in sub-Saharan Africa (Lopez et al., 2006).

Immunopathology of Schistosomiasis

Immunity to Schistosomiasis in Animal Models:

There are many animal models for schistosomiasis. The most useful models for human disease are mice, non-human primates and other mammalian hosts like cattle, buffalo, goats and pigs. A great deal of the information about the mammalian immune response to schistosomes has been generated using B-cell-deficient and IFN-γ knockout mice or double (IL-4 and IL-10 deficient or IL-10 and IL-12 deficient) knockout mice (Freeman et al., 2006). Other informative work has been conducted using immunization with UV- or γ -irradiated cercariae in mice, nonhuman primates, or other mammals (Bickle et al., 2001). Irradiated larvae do not mature into adult worms so they cannot lay eggs. As a result, these models are not 'complete' because there is no egg-induced liver pathology. Another interesting model is artemether-treated mice that subsequently show high-level resistance against schistosome re-infection (Bergquist et al., 2004). This model may be particularly useful for understanding protective mechanisms against re-infection. Artemether is effective mainly against schistosomula, adult worms are largely refractory to the drug. The artemether-affected worms show vesiculation, peeling of the tegument, and atrophic testes and ovaries (Xiao & Catto, 1989).

During the acute stage of murine schistosomiasis, stimulation of the Th2 response by egg antigens leads to increased serum IL-5, massive eosinophilia in bone marrow and blood, and granuloma formation around the egg, with collagen deposition, and tissue fibrosis. Eosinophils form the majority of cytokine producing cells in the granuloma and are the dominant source of

IL-4 (Rumbley et al., 1999). The role of eosinophils in the mouse model of infection is still unclear (Swartz et al., 2006).

The chronic stage of murine schistosomiasis is predominantly Th1- mediated, largely based on an increasing ratio of Schistosome-specific IgG2a to IgG1, reduced eosinophil and IgE levels (Borojevic, 1992), and diminished Th2 responses (Henderson et al. 1992). The Th1-Th2 balance in murine schistosomiasis is regulated by IFN-γ, IL-4 and IL-10, with IL-4 driving the reaction in the direction of Th2 and IFN-γ driving it toward Th1, while IL-10 may inhibit either trend depending on the situation (Boros & Whitfield, 1998).

Although generally considered to be a good model, there are still some controversial findings regarding the biological role of IgE in experimental murine *S. mansoni* infection (Amiri et al. 1994). IgE is supposed to represent a major defence component against schistosomiasis, probably by targeting effector cells to the invading cercariae in antibody-dependent cell-mediated reactions. However, murine *S. mansoni* infection is not associated with detectable serum level of IgE until maturation of the worms, pairing and deposition of eggs (Sher et al., 1990).

Immunity to Schistosomiasis in Humans:

As outlined above, schistosomes are complex parasites with several distinct life stages (cercaria, schistosomula, adult, and egg) in different anatomical sites of its definitive host (Gryseels, 2012). Each of these stages express hundreds, if not thousands, of antigens, many of which would be expected to elicit an immune response. The human immune response to schistosomes includes innate and adaptive stages (Colley & Secor, 2014). During initial invasion and migration stages of the parasite life cycle (ie: first infection), the immune response is predominantly innate, followed quickly by a Th1-dominant adaptive response (Hagen et al.,

2015). Innate immunity is reflected in the number and cytotoxic activity of natural killer (NK) cells that can be a source of IFN-γ and mediate antibody dependent cell mediated cytotoxicity (ADCC) (Comin et al., 2008). Comin et al., 2008 also suggested that one or more toll-like receptors (TLR) may mediate the interaction that triggers the production of IFNγ by NK cells. Several human studies (Nishioka et al., 2001 and Yu et al., 2001) demonstrated that dendritic cells (DC) can stimulate the cytolytic activity of NK cells. Another study using human peripheral blood monocytes showed bidirectional activation between NK cells and DCs (Gerosa et al., 2002). Several serine proteases with elastase-like activity are produced by the acetabular glands. These cercarial secretions are the first parasite molecules encountered by the host immune system (Minard, 1977; Salter, 2002; Stirewalt, 1974).

Adaptive responses are relatively well-characterized. Following cercarial penetration, an IgE-mediated hypersensitivity response can occur against the cercariae (dermatitis) (Gryseels et al., 2006). In the acute stage of the disease, human patients develop a T helper 1 (Th1) mediated immune response, characterized by high levels of interferon (IFN)-γ (Henri et al., 2002). Following parasite maturation and egg production; Th1 responses are down-regulated and a strong Th2 response emerges that is characterised by high production of IL-4, IL-5 and IL-13, as well as IgE synthesis (Montenegro et al., 1999). When serum IL-5 level increases, this results in massive bone marrow and blood eosinophilia. Eosinophils accumulate in the developing liver granulomata as a site of active inflammation and tissue remodeling. Several eosinophil products have a role in debris removal and tissue remodeling activity including eosinophil peroxidase (Horton, 1996), ribonucleases (Rosenberg, 1998), matrix metalloproteinases, (Schwingshackl, 1999) and the protease inhibitor, plasminogen activator inhibitor-2 (PAI-2) (Swartz et al., 2004).

Another important role of eosinophils is secretion of endogenous IL-4 that has an important role in maintaining the Th2 response to infection (Sabin 1996; Shinkai et .al., 2002).

Maintaining a balanced and controlled Th1 and Th2 response ensures formation of protective granulomas around parasite eggs without excessive pathology. Formation of granuloma around the eggs, mainly in the liver, as well as in the lung, pancreas and lymph nodes result from Th2 activation (Caldas et al., 2008). The granuloma consist mainly of eosinophils, T cells and macrophages (Pearce and MacDonald, 2002). In the chronic stage of the disease, the Th2 response is down-regulated by IL-10 leading to a reduced cellular response to schistosome antigens (Caldas et al., 2008). For that reason, most chronically-infected patients are asymptomatic (Caldas et al., 2008).

The parasite has many strategies for evasion the host immune system, resulting in a chronic infection (Wilson et al., 2004). These include the schistosome's capability to continually regenerate its outer tegument through unique somatic stem cells (Jenkins et al., 2005; Lightowlers & Rickard, 1988), produce antigens that are similar to endogenous host components (Yoshino & Bayne, 1983; Salzet, 2000), and antigen 'disguise' through acquisition of host molecules to cover the outer worm surface (McLaren, 1984; McLaren & Terry, 1982).

Immunology of resistance to infection in humans

Putative resistant (PR) individuals, who resist natural schistosomiasis infection in spite of living in endemic areas are of great interest for vaccine developers (Tran et al., 2006). This group is defined as follows:

1- Fecal egg counts are negative despite more than 5 years of *S. mansoni* exposure.

2- They have not been treated for schistosomiasis.

3- Their exposure to infection is continuous and not interrupted.

4- Their cellular and humoral responses are strong to crude schistosomal antigens. (Correa-Oliveira et al., 2000;Correa-Oliveira et al.,1989;Viana et al,1995 ; Viana et al.,1994).

A comparison of the immune responses of PR individuals and chronically infected patients to adult worms and crude extracts from schistosomula revealed striking differences (Caldas et al., 2000; Viana et al, 1995; Viana et al., 1994). PR individuals respond to these antigens with both Th1 and Th2 type cytokine responses (Caldas et al., 2000), but chronically-infected patients show Th2 type responses only (Roberts et al., 1993). Schistosomulum antigens that stimulate Th1 responses (mainly IFN-γ) may mediate resistance in the PR individuals (Correa-Oliveira et al., 2000). SmTSP-2 (Tran et al., 2006) and Sm29 (Cardoso et al., 2006) are candidate vaccine antigens present in the *S. mansoni* tegument that were identified using sera from PR individuals. It has been suggested that the PR immune response can be used as a guide to select tegumental proteins for inclusion in a multivalent recombinant vaccine (Loukas et al., 2007).

Schistosomiasis Control

- **Treatment of Schistosomiasis**

Schistosomiasis control efforts currently focus on the reduction of morbidity through periodic treatment with praziquantel (PZQ) (WHO, 2002). However, chemotherapy does not prevent re-infection. For the effective reduction of schistosomiasis morbidity and mortality; we must recognize that morbidity, transmission, and infection are different aspects to be controlled and that mass PZQ chemotherapy is likely inadequate to reach the goal of control (Engels et al., 2002; Bergquist et. al., 2005). PZQ has only moderate action against immature worms so a second dose is needed after a short period to eliminate newly matured worms (Doenhoff et al. 2008). Mass treatment of all parts of an endemic area can be so expensive that it is impractical in many low-income countries (McManus & Loukas, 2008). In the past 10 years, resistance has

emerged to praziquantel (McManus & Loukas 2008). Therefore, chemotherapy alone is unlikely to reduce infection levels over the long term. Thus, several practical approaches have been suggested to augment treatment programs including reducing exposure to contaminated water, decreasing availability of the intermediate snail host, or preventing snail infections by reducing water contamination with egg-containing excreta (Evan Secor, 2014). Of course, the development of an effective vaccine would contribute enormously to the reduction of the burden of disease attributable to schistosomiasis. As a result, combined vaccination and chemotherapy are likely to be necessary to achieve the WHO goal of control (Bergquist, et al., 2002). Miyasato et al., 2009 have recently reviewed the rationale that underlies the global effort to develop schistosomiasis vaccines:

• Vaccine-induced protection can be achieved in experimental animals

• Vaccination can help avoid retreatment for re-infection

• Expanded chemotherapy programs increase the risk of drug-resistance

• Reduction of infection intensity in adolescents and older age groups compared to children suggested the possibility of acquired (partial) immunity

Schistosome Biology

Many experimental difficulties are encountered by those who want to study schistosome biology (Wilson et al., 2007). The first difficulty is the complex life cycle of the parasite, including two different hosts and many different life-stages. Schistosomes are able to live in both vertebrate and invertebrate hosts, as well as spending short periods as free-living organisms (eg: cercariae and miracidia). The second difficulty is the inability to achieve parasite multiplication *in vitro* in spite of the fact that they can be cultured for prolonged periods (Basch, 1981). The

only reliable source of parasites is infected laboratory snails/animals from which they are isolated, a factor that greatly limits the number of parasites available for experimental studies.

Diagnosis of schistosomiasis

The recent World Health Organization (WHO) road map statement emphasized the provision of regular treatment for at least 75% of children at risk by 2020 to reduce the global impact of schistosomiasis, with the aim of disease elimination (World Health Organization, 2012). Accurate diagnosis and quick treatment during the initial stages of schistosomiasis can cure the disease without the development of complications. The global disease burden of schistosomiasis is still high (Mutapi, 2015), at least in part due to the lack of good diagnostic tests for schistosome infections in endemic areas (Weerakoon, 2015). Therefore, the development of better diagnostic methods to identify this infection would be of great benefit.

A number of diagnostic techniques have been used for schistosomiasis, starting with basic microscopic detection of eggs in stool or urine samples and, more recently, the development of more sophisticated approaches. The current diagnostic tools for schistosomiasis can be grouped into the following four main methods: (1) direct parasite detection (2) immunological diagnosis; (3) DNA and RNA detection; and (4) biomarkers.

Direct parasite detection methods

- **Microscopic examination:** Microscopic examination for the presence of eggs in stool or urine samples is the most straightforward and widely used diagnostic method to test for schistosome infection (Utzinger et al., 2011). The Kato-Katz technique (Katz et al., 1972) is the most practical method for finding eggs in stool samples, while filtration and centrifugation are used for the diagnosis of urinary schistosomiasis. These archaic methods are considered to be the "gold standards" in highly endemic regions. Since shedding of eggs

is irregular, three different samples are typically required for optimal detection of eggs. This is further complicated by the fact that this method is time-consuming and labour-intensive (Zhou et al., 2011). In short, microscopic examination of stool or urine can be highly specific but lacks sensitivity.

- **Organ biopsies:** Rectal or bladder biopsies for the identification of eggs can be performed if stool or urine tests are egg-negative (Wu et al., 2012). This technique is valuable in the clinical management of patients, particularly in cases where the clinical evidence is suggestive of schistosomiasis but cannot be confirmed by other diagnostic tests (Gray et al., 2011).

Immunological diagnosis

Immunological tests detect anti-schistosomal antibodies or circulating schistosomal antigens in plasma, serum, urine, or sputum (Gray et al., 2011). Immunological methods are used when the parasitological tests are negative, particularly for patients in low prevalence locations (De Noya et al., 2007).

- **Immunological methods for antibody detection:** Various serodiagnostic methods have been developed to detect anti-schistosomal antibodies. The most commonly used techniques include enzyme-linked immunosorbent assays (ELISA), the circumoval precipitin test (COPT), the indirect hemagglutination test (IHAT), the indirect immunofluorescence test (IFT) and the skin reaction test. These tests are typically reproducible, sensitive, and time-saving compared to microscopic methods. However, they are relatively expensive, trained technicians are required to perform the tests, cannot distinguish between active and past infections, are relatively nonspecific and are liable to cross-reactions with other antigens (Oliveira et al., 2005; Ross et al. 2002; Stothard et al.,

2009). Additionally, after treatment, positive antibody tests might take a very long period of time to become negative because they are based on the determination of the humoral immune response (Abbasi, et al., 2010; Doenhoff, et al., 2004; Foo et al., 2015; Wang et al., 2008). Finally, immunological methods cannot measure the intensity of infection (Doenhoff, et al., 2004).

- **Immunological methods for the detection of parasite-derived circulating antigens:** These methods are an effective way to differentiate active and past infections. A variety of schistosome-specific antigens are released into the circulation of the host due to the regurgitation of gut content by adult worms. Deelder et al. (1976 & 1989) have reported the presence of both a negatively charged anodic antigen (CAA) and a positively charged cathodic antigen (CCA). Several studies have used monoclonal antibodies to detect and quantify these two antigens in serum and urine of infected individuals (Deelder et al., 1980; Stothard et al., 2006; Van Dam et al., 2004; Van Lieshout et al., 2000). Despite having low sensitivity in low prevalence locations, these assays have been used successfully in endemic areas with moderate and high prevalence of schistosomiasis (Van Dam et al., 2004; Van Lieshout et al., 2000). Corstjens et al. (2008) and Stothard et al. (2006) have both developed dipstick qualitative tests for the detection of CCA. This test is simple, rapid and can be easily implemented in the field. In addition to CAA and CCA, there are other candidate antigens that are secreted by schistosome eggs (Asahi & Stadecker, 2003; Boros, 1999). Some of these antigens can be detected by ELISA using monoclonal antibodies that recognize specific glycan motifs (Nourel Din et al., 1994).

DNA and RNA Detection based methods

Schistosome DNA or RNA detection by conventional or more advanced Polymerase Chain Reaction PCR-based techniques, such as real-time quantitative PCR or multiplex PCR are promising additions to the parasitological and serological tests for schistosomiasis (Weerakoon, 2015).

- **Detection of Schistosoma DNA by PCR:** Schistosomal DNA derived from dead worms, tegument shedding or inactive eggs can be found in host serum and other tissue samples (Xu et al., 2013). Therefore, PCR has become a promising diagnostic alternative to overcome the weaknesses of microscopic methods. Conventional PCR amplifies a precise target gene segment and is a specific method for the direct recognition of schistosome DNA in stool samples (Lier et al., 2006; Sandoval et al., 2006). Real-time PCR (qPCR) has advantages compared to conventional PCR: such as, its ability to detect target DNA at lower concentrations, it is quantitative, and since there is no need for electrophoresis to visualize products, it is less labor-intensive. PCR methods can have high sensitivity and specificity, but their use in epidemiological studies is limited. Compared to the Kato-Katz technique, PCR requires more advanced equipment and technical effort. However, because of the recent development of DNA isolation procedures and PCR technology, particularly real-time PCR, molecular diagnosis has become a good alternative to microscopic examination in some settings (Espy et al., 2006; Klein, 2002; Verweij et al., 2004; Verweij et al., 2007).
- **Detection of Circulating miRNAs**

  MicroRNAs (miRNAs) are a group of noncoding RNAs that are involved in posttranscriptional gene regulation (Bartel, 2004). They are present mainly in body fluids, such as blood plasma/serum. They are released from cells into the circulation through

three main pathways: leakage from broken cells, active secretion of miRNAs enclosed in exosomes, as well as active secretion of miRNAs bound to RNA binding proteins (Chen et al., 2012). Identification of parasite-derived miRNAs in both *S. mansoni* (De Souza Gomes et al., 2011) and *S. japonicum* (Cai et al., 2011) provided the basis for their detection in the circulation. The identification of schistosome-specific miRNAs was first reported in the plasma of *S. japonicum*-infected rabbits (Cheng et al.,2013), followed by report of elevations of several parasite-derived *S. mansoni* miRNAs in a mouse model (Hoy et al., 2014). He et al., 2013 studied the serum levels of host miRNAs in mice, rabbits, buffaloes, and humans infected with *S. japonicum*, and suggested that circulating miR-223 is a potential new biomarker for the detection of schistosome infection as well as the assessment for chemotherapy response. However, host-derived miRNAs cannot differentiate between *S. mansoni*-infected and uninfected individuals (Hoy et al., 2014). Nonetheless, the identification of schistosome-specific and infection-associated host miRNA profiles have potential as diagnostic markers of early infection, in the evaluation of disease progression, and in monitoring therapeutic responses. However, they are limited in their wide-scale application because they need to be applied in clinical settings. Another limitation is the costs of the required reagents and the technical resources (Weerakoon, 2015).

Biomarkers for schistosomiasis

In 1998, the National Institutes of Health defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definition Working Group Biomarkers and surrogate endpoints, 2001; De Gruttola et al., 2001). The

progress of molecular biology technologies, such as cDNA microarrays and high-throughput mass spectrometry (MS)-based proteomics, have revolutionized the field of biomarker discovery. In particular, the latter makes it feasible to use a very sensitive technology to detect and quantify proteins in blood (Iskandar & Ciorba, 2012; Marshall et al., 2014). In parallel, advances in bioinformatics have improved the ability to collect, characterize and analyse large amounts of data (Cioffi et al., 2015). The sequencing of the human genome revealed 20,000 to 25,000 genes (International Human Genome Sequencing Consortium, 2004). Each gene has the potential to encode many proteins, therefore, a huge number of different proteins are produced in the human body. As a result, studying genes alone is not enough to solve the different mysteries of disease. Proteomics is a science that has emerged after the genomic revolution (Persidis, 1998), which deals with protein identification, post-translational modification (PTM), and protein-protein interactions in complex biological systems. Understanding of protein functions, expression and interactions is crucial in the pursuit for information regarding life and disease. One of the most important applications of proteomics is the discovery and identification of biomarkers for disease (Jones et al., 2002).

However, in the field, this method has limited applications. Non-invasive biological markers, serum antibodies, cytokines, and circulating microRNAs provide promising ways to diagnose schistosomiasis (Olveda et al., 2014; Dos Santos et al., 2005; Rossi et al., 2007). Research on circulating antigens has focused on two antigens derived from the schistosome gut: CAA and CCA. Schistosome CAA and CCA are excreted by the kidney into the urine at amounts detectable by antigen capture methods (Stothard, 2009). Several studies have observed the elevation of alkaline phosphatase and gamma glutamyl transpeptidase (GGT) levels in schistosomiasis (Barreto, 1971; Vianna et al., 1989). Mansour et al. (1982) reported that GGT

levels were elevated in the mild forms of schistosomiasis due to the migration of eggs to the liver and granuloma formation. They also noticed that the level of GGT was higher in the case of hepatosplenic schistosomiasis with gastrointestinal bleeding. Manivannan et al. (2010) studied different liver protein patterns in mice with hepatosplenic schistosomiasis using differential in-gel electrophoresis and recorded 80 protein spots that changed significantly with infection. Of these changed spots, 35 were related to severe disease.

Many studies on *Schistosoma*-infected patients have reported that higher parasite burdens and parasite susceptibility are associated with higher levels of anti-*Schistosoma* IgG4 in infected children (Hagan et al., 1991; Grogan et al., 1996; Naus et al., 1999; Naus et al., 2003). Additionally, Naus et al. (2003) showed that the reactivity of anti-SWAP (adult worm antigen preparation) IgE increases with age in endemic areas of schistosomiasis.

Despite considerable effort and the wide range of tests outlined above, reliable biomarkers for early disease and low-intensity *S. mansoni* infection are not available. New biomarkers are also needed to track disease complications (see below) and the success of therapeutic interventions (ie: tests of cure).

Diagnosis and evaluation of schistosome-induced liver complications

Various diagnostic methods are used to evaluate the complications of schistosomiasis (mainly liver fibrosis).

- **Liver biopsy:** Biopsies can directly detect hepatic damage and is still the gold standard method. However, it is invasive and causes discomfort, is prone to sampling error and post-procedural risk and is clinically impractical in the field (Shiha et al., 2009; Sinkala et al., 2015). In *S. mansoni*, a wedge biopsy of the liver is the most accurate method to assess fibrosis. However, it requires major surgery. Since the intensity of periportal

fibrosis varies throughout the hepatic parenchyma, a fine-needle biopsy does not always give a true histopathological picture (Dimmette, 1955).

- **Imaging:** In hospitals, imaging has proved to be an important tool for assessing hepatic fibrosis. In particular, ultrasonography is an important assessment tool because it can measure liver enlargement and periportal thickening (Domingues et al., 1993; Richter et al., 2001; Vezozzo et al., 2006). The limitations of imaging are its dependence on operator skills. Another problem is the reported interobserver variation when doing quantitative measurement (Pinto-Silva et al., 2010; Skelly, 2013).
- **Biomarkers**: Even though there are many good markers of fibrosis, until now these markers have not been good enough to replace ultrasonography when evaluating fibrosis in schistosomiasis (Domingues et al., 2011). Important markers of fibrosis have been reported in many studies. One of these is the inverse correlation between the thickness of the gallbladder wall and either anti-SEA (soluble egg antigens) or anti-SWAP IgE (Negrão-Corrêa, et al., 2014). An elevated serum level of IgG in *S. mansoni* patients is correlated with the progression of liver fibrosis (Henrique et al., 2009). Others, studying the relationship between fibrosis and inflammatory makers in a case-control study of schistosomiasis, have demonstrated that hyaluronan and laminin are higher in patients with hepatosplenic schistosomiasis compared to controls. Elevation of these markers suggested they may be useful in diagnosing and monitoring periportal fibrosis (Sinkala et al., 2015). Pereira et al., 2015 reported that schistosomal egg antigens directly induce host liver cells to proliferate and produce the pro-fibrogenic molecule called osteopontin. They also proved that serum and hepatic osteopontin levels correlate with liver myofibroblast accumulation and liver fibrosis stage in mice and human, and a direct

measure of portal hypertension. As a result, osteopontin is also a possible non-invasive biomarker in *S. mansoni* infection (Pereira et al., 2015).

Proteomic Studies

Proteomic analysis is a multistep procedure including preparation of the sample, separation, quantification, identification of proteins and finally validation of candidate biomarkers (Shevchenko et al., 2015). Nonspecific detection methods such as centrifugation, chromatography, dialysis, electrophoresis, and immunoprecipitation, are all classical methods of proteomic analysis that are labour intensive and need large volumes of each sample. Nowadays, these methods are replaced by specific detection methods such as indirect antibody-based immunoassays such as enzyme-linked immunosorbent assay (ELISA) and, more recently, mass spectrometry (MS). MS is potentially very well-suited for clinical laboratories. Although MS is predicted to replace immunoassays for many routine protein analyses, relatively few protein MS applications are currently in routine clinical use (Scherl, 2015). Any MS essentially consists of three basic parts, **an ion source**, **a mass analyzer** and **a detector** (Bensimon et al., 2012; Gstaiger & Aebersold, 2009; Herrero et al., 2012; Mishur, & Rea, 2012; Yates et al., 2009). In the ion source the analyte is ionized (converted into gas-phase ions). Several ionization methods have been described, such as electron impact, fast atom bombardment, chemical ionization, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The last two are so-called 'soft' ionization techniques, i.e. ionization of the analyte without fragmentation. Once in the gas phase the analytes are separated based on their mass-to-charge (m/z) ratios in the mass analyzer. Although many types of mass analyzers can be used either in clinical laboratories or for research applications, so-called quadrupole and ion trap machines are

the most commonly used today (Scherl, 2015). Other machines including time-of-flight, ion cyclotron and orbitrap analyzers are also available.

- The quadrupole mass analyzer (mass filter): consists of four parallel voltage carrying rods (electrodes) in which the ions pass from one end to another end. This method uses oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through the system. Only ions at unique m/z ratio have a stable trajectory and pass across the quadrupole at any time while ions at other m/z values hit the electrodes. The oscillating electrical fields can be changed rapidly to scan a wide mass range (Siuzdak, et al., 2004; Maher et al., 2013).
- Quadrupole ion trap analyzers: works on the same physical principles as the quadrupole mass analyzer, but unlike to quadrupole mass analyzer the ions introduced to the mass analyzer in a pulsing mode. A linear quadrupole ion trap is similar to a quadrupole ion trap, but the trap can store large volumes of ions and it has high sensitivity (March, 2000).
- Time-of-flight (TOF) analyzers are field-free tubes where ions travel after an acceleration using an electrical field pulse. The same kinetic energy is applied to all ions, and then their flight time toward the detector is measured and converted into an m/z value. Ions with low m/z value will travel faster and reach the detector first (Guilhaus, 1997).
- Fourier-transform-ion cyclotron resonance analyzer: ions move in a circular path in a magnetic field that represents their m/z values in ion cyclotron resonance mass analyzer. When energy is provided in a direction orthogonal to their plane of precession and at a frequency equal to their precession frequency, the ions are accelerated perpendicularly to the field. This acceleration induces a cyclotronic motion. The ions will impact the wall of

the trap, where the detector is located, enabling the image current of this cyclotronic motion to be detected. Then transformed into frequency values using Fourier-transformation, and eventually converted into m/z values (Marshall et al., 1998).

- Orbitrap mass analyzer: it is a non-magnetic mass analyzer but it consists of two electrodes inner spindle and outer barrel that created together an electrostatic field to separate the masses. The Orbitraps that are commercially available are LTQ Orbitraps. The Orbitrap gained more attention in the proteomic field when it was introduced because of its high mass accuracy, high sensitivity and a high dynamic range (Hu, 2005).

All mass analyzers have diverse performance characteristics. Their performance measurements are resolution and mass accuracy. The resolution is the capability to discriminate two closely peaks. Resolution is calculated as the ratio of the m/z value of a compound divided by the full width at half maximum (FWHM) of the peak. Therefore, resolution is a ratio, without units. Mass accuracy is the difference between the theoretical mass of a compound and the measured mass. Mass accuracy is stated in one of two unites, absolute m/z units (Da) or in relative units parts per million (ppm) (Scherl, 2015).

In this thesis, I briefly describe recent advances in bioanalytical approaches for biomarker discovery. These approaches focus on blood samples (serum). Protein separation is done using either zoom fractionation or ion exchange fractionation. Classical proteomics methods for protein identification, which used SELDI-TOF (Surface-enhanced laser desorption/ionization time-of-flight), MALDI -TOF MS (Matrix assisted laser desorption/ionization time of flight) or ESI (Electrospray ionization as sources for mass spectrometry, and up-to-date mass analyzers (e.g. Orbitrap) are also used. This information can be used to identify proteins, which may enable new diagnostic strategies.

Techniques

SELDI:

The main components of this technology are the ProteinChip Arrays, the ProteinChip Reader, and the accompanying software. ProteinChip Arrays are composed of twelve eight-spot chips gathered in 96-well (bioprocessors). The proteins that are captured on the chips can be analysed and detected by the ProteinChip Reader. Relative expression levels of proteins at certain molecular weights are compared by a variety of statistical techniques and bioinformatic software resulting in a 'pattern' of proteins characterized by mass-to-charge ratio (m/z) (Reddy & Dalmasso, 2003). ProteinChips with different affinity surfaces like copper (IMAC), hydrophobic, cation exchange, anion exchange, and silica coated chips have been used with SELDI to reduce the complexity of samples (Merchant & Weinberger, 2000). SELDI method has recently been developed for a higher throughput in clinical settings (Popescu, 2014). Several researchers have provided evidence that this platform can be used to uncover proteomic expression patterns associated with a disease state (Engwegen, 2006). SELDI can be used to analyze large numbers of small-volume samples. In addition, SELDI is more adapted for less than 30 kDa molecular mass proteins (Atanassov, 2015). Even though SELDI-MS platform led to the improvement of biomarker discovery, it has some limitations. One of these is the restriction of the number of compounds under examination because only a fraction of the peptides/proteins bind to the different matrixes (Vlahou, 2001; Chapman, 2002; Diamandis, 2004). Also, peaks below 30 kDa are particularly well-resolved with this method, this is not the case for higher molecular weight proteins (Seibert et al., 2004). Another limitation is that the system is expensive and demands well-trained laboratory workers (Paweletz et al., 2001). Finally, while the identification of proteins is very important, the focus of SELDI technology is

directed towards investigating protein differential expression (protein patterns) (Seibert et al., 2004).

Some examples of the application of SELDI are:

Oncology: Several profiles of proteins and biomarkers in many types of cancer have been identified for potential use in the early detection of prostate, breast, ovarian, and other types of cancer (Gallo et al., 2016; Petricoin et al., 2002).

Infectious diseases: Biomarkers of infectious diseases such as HIV (Ratto et al., 2008), Severe Acute Respiratory Syndrome (SARS) (Kang et al., 2005 & Poon et al., 2004), hepatitis B and C (Cui, et al., 2007 & Schwegler, et al., 2005), bacterial endocarditis (Fenollar et al., 2006) and parasitic infections such African Trypanosomaisis (sleeping sickness) (Papadopoulos et al., 2004) or trematode Fascioliasis (Rioux et al., 2008) have been identified. For example, using Ciphergen's PCI-1000 ProteinChip Interface on a tandem MS system, alpha-defensin-1, -2, and -3 were detected as novel inhibitors of HIV-1 replication (Zhang L, et al. 2002).

Immunology: Using the SELDI-MS platform, two independent studies have reported particular urinary biomarkers associated with the rejection of renal allografts (Clarke et al., 2003; Schaub et al., 2004).

In this thesis work, SELDI-TOF MS was used to screen serum samples from 85 human samples (30 schistosome infected patients and 55 control) to identify biomarker peaks for the diagnosis schistosomiasis and to differentiate between *S. haematobium* and uninfected controls. SELDI-TOF MS was also used to study serum protein expression patterns. This was done using 26 CD1 mice that were infected with different numbers of *Schistosoma mansoni* cercariae, as well as 26 inbred mice C57Bl/6 that were infected with 200 *S. mansoni* cercariae.

MALDI -TOF

MALDI-TOF mass spectrometry has been widely used for the analysis of high molecular weight biomolecules such as proteins (Scherl, 2015). In MALDI, the analyte is loaded into the solid matrix, this leads to its crystallization with the matrix, and introduced in solid form into the mass spectrometer. After the crystallization of the matrix and sample, the target is bombarded with brief laser pulses to form gaseous ions. The matrix is protonated first and consequently protonates the analyte during the MALDI process (Knochenmuss, 2006). The matrix composition varies depending on the analyte biomolecule and the type of laser applied (Fenselau & Demirev, 2001). According to protein or peptide profiling and analysis, sinapinic acid (SA) is the most commonly utilised matrix for proteins analysis whilst peptides are thought to ionise more effectively using cyanohydroxycinnaminic acid (CHCA) as the matrix. Both of SA and CHCA are used in concentrations of ~10 mg/mL and thus represent an excess of matrix molecules to analyte molecules in the resulting mixture. Therefore, the lower end of the mass spectrum (less than 500 Da) is represented by ions arising from the matrix and is usually not studied during analysis. More matrix materials have been investigated in order to gain improvements in ionisation efficiency such as, 2, 5-dihydroxybenzoic acid, 2, 4-hydroxy-phenyl benzoic acid, and Ferulic acid (Fenselau & Demirev, 2001; Giebel et al., 2010; Vaidyanathan et al., 2002; Williams et al., 2003). However, the organic matrix molecules are evaporated and ionized at the same time as the analytes, and mass peaks of the fragmented matrix molecules are produced. The heterogeneity in matrix–sample crystal formation, makes the mass peaks of the fragmented matrix molecules irreproducible, resulting in a different pattern at each measurement. Therefore, it is very difficult to separate the mass peaks of the analytes at the low m/z range, and the analysis of small molecules is very restricted using MALDI-TOF mass spectrometry (Kang et al., 2005; Wei et al., 1999). Matrix clusters might be reduced by:

1) Purifying samples on reverse-phase (RP) microcolumns. However, some peptides might be lost on RP columns (Guo et al., 2002).
2) Using cetrimonium bromide in the matrix solution (Guo et al., 2002). Although it is very efficient in suppressing matrix clusters, it lowers the intensity of sample ions.
3) Recrystallizing the matrix in ethanol (Keller & Li, 2000).

However, MALDI-TOF has many advantages, such as easy sample preparation, high sensitivity (fmol) and a wide detection range (600kDa) (Kim, 2015). MALDI also allows for rapid sample analysis and is tolerant of heterogeneous samples such as proteolytic digests (Valaskovic, et al., 1995). MALDI-TOF has the additional advantage of direct identification of peaks of interest by analyzing samples in TOF/TOF mode (Orvisky et al., 2006). The identification of native peptides is more difficult than identification of peptides resulting from tryptic digestion. As a result, the search algorithms typically used for the identification of tryptic peptides may not be suitable for the analysis of native peptides (eg: Sequest, Mascot, Sequest–PeptideProphet, Spectrum Mill, Sonar, or X!Tandem). Post-translational modifications (PTMs) are another obstacle for identification (Mischak et al., 2009; Theodorescu & Mischak 2007).

Applications of MALDI:

The capability of MALDI-TOF MS to yield rapid microbial identification favors potential applications in many fields, such as medical diagnostics, food quality control, and environmental monitoring (Croxatto et al., 2012). MALDI-TOF-MS has proved itself in the fast and reliable identification of microorganisms such as pathogenic bacteria and yeasts (Ryzhov & Fenselau, 2001; Holland *et al*., 1999; Fenselau & Demirev, 2001; Sun *et al*., 2006; Dieckmann *et al*., 2008).

In this thesis, several differentially-expressed host proteins were identified in the serum of *S. mansoni* infected mice at different disease stages using MALDI TOF MS.

ESI

ESI is a very powerful technique capable of analyzing small and large molecules of various polarities in a complex biological sample. ESI is valuable in the characterization and identification of nucleic acids and proteins (Laughlin & Wilson, 2015). In ESI, the analyte solution is injected by a mechanical syringe pump through a hypodermic needle or stainless steel capillary into the ion source. A high voltage (2–6 kV) is applied to the tip of the metal capillary, located at 1–3 cm from the spray needle tip. The strong electric field results dispersion of the sample solution into a highly charged droplets. During the gas phase, the ions are directed toward a mass analyzer and detector, and their m/z value is determined (Scherl, 2015). The development of 'soft' ionization techniques such as ESI-MS has found widespread application in structural biology. ESI-MS is a method of choice for the characterization of macromolecules because of its ability to transfer large biomolecular complexes intact into the gas-phase, combined with low sample consumption and high sensitivity (Fenn, 2003; Hopper & Robinson, 2014; Kebarle & Verkerk, 2009; Rajabi et al., 2015).

Even though both ESI and MALDI can generate [M + zH] z+ ions, it is more common for MALDI to form single charged ions (z = 1) and for ESI to form multiply charged ions ($z \gg 1$) (Nilsson, 2010). Using ESI mass spectrometry as an analytical method has many advantages such as its ability to be coupled to high-performance liquid chromatography for molecular fractionation before analyzing the sample by mass spectrometry (Nilsson, 2010). The small m/z ratio range of the quadrupole mass analyzer makes it possible to identify the mass of the sample

with a great precision (ie: mass accuracy). A final advantage of ESI is its high sensitivity which makes quantitative and qualitative measurements more accurate.

One of the disadvantages of ESI mass spectrometry is the difficulty encountered when cleaning the apparatus as it is prone to contamination from previous experiments. Also, multiply charged ions can lead to confusing spectral data and this becomes even more complicated when using a biological samples.

Applications of ESI:

- ESI-MS reports on biological systems have involved double helical DNA studies, establishing conditions for preserving the duplex in the gas phase (Light-Wahl et al., 1993). Several reports on duplex stability have been published (Gabelica et al., 2002; Gidden et al., 2004; Light-Wahl et al., 1993). Dissociation to single strands as well as nucleic acid sequence studies have been reported (Barylyuk et al., 2013; Ni & Chan, 2001; Fabris, 2011; Madsen & Brodbelt, 2010; Wan et al., 2000).
- ESI has the ability to identify organisms directly from patient specimens without culture. This is helpful for the detection and identification of organisms even at a very low concentration (Buchan & Ledeboer, 2014). Butler-Wu et al., (2011) have found a poor recovery of organisms from joints suspected to be harboring bacteria when routine culture methods are employed. On the other hand, ESI-MS identified a likely pathogen in 100% of cases when doing a clinical diagnosis of prosthetic joint infections (Jacovides et al., 2012).
- ESI-MS has been used to identify filamentous fungi in sputum specimens (Simner et al., 2013).

- ESI-MS has also been applied to identify bacterial pathogens from a variety of samples, such as food, cell cultures, and environmental samples (Sampath et al., 2012).

In this thesis, several distinct candidate biomarkers were identified using ESI that could distinguish schistosomiasis from controls in 85 human samples (30 *S. haematobium* infected patients and 55 control).

LTQ-OrbitrapVelos

The continued development of mass spectrometers, including Orbitrap-based systems was driven by objectives of directly analyze complex samples without extensive pre-fractionation as well as increasing sensitivity and accelerating scan speed to meet the evolving needs of proteomics researchers (Eliuk & Makarov, 2015). The LTQ Orbitrap Velos hybrid mass spectrometer was generated with capabilities aimed directly at accomplishing these objectives (Olsen et al., 2009). Orbitrap-based mass spectrometer is Orbitrap mass analyzer equipped with an electrospray ionization source (ESI) (Hu, 2005). LTQ-Orbitrap Velos machines have been used in a huge range of experiments such as metabolomic analyses and clinical proteomics (Helfer et al., 2015; Kim et al., 2010; Zhao et al., 2016). The Orbitrap resolving power of this method exceeds 60'000 FWHM at 380-1400 m/z with a wide dynamic range. The dissociation methods of LTQ-Orbitrap include collision induced dissociation (CID), pulsed-Q-dissociation (PQD) and higher energy collision dissociation (HCD).

The fragmentation process in CID depends on the facilitation of collisions between the inert background gas, such as helium and a precursor ion. Once the collision energy is high enough, the fragmentation of the excited ion occurs (Wong et al., 2009; Rohmer et al., 2011). However, due to the resonant excitation principle, the fragmentation process in CID is weak. Therefore, m/z ions with the lowest stability can become trapped in the ion trap. Many

techniques have been introduced to overcome or reduce this limitation. One of these is PQD which is a modified resonant excitation technique that originated from CID but provides access to low m/z ions (Rohmer et al., 2011). In PQD, the activation is dependent on an increase in the collision voltage that is achieved in a short period (Cunningham et al., 2006). With the introduction of hybrid instrumentation, such as HCD in Orbitrap-based instruments, another complementary excitation technique has been developed (Makarov, 2000; Makarov et al., 2006). This hybrid instrument allows both conventional CID and PQD resonant modes, and the current HCD non-resonant mode to be combined (Hager, 2002).

Applications of LTQ-OrbitrapVelos:

- The LTQ Orbitrap has been used to analyze intact small (3–10 kDa), medium (10–25 kDa) and large (150 kDa) proteins (Thevis & Schänzer, 2008; Waanders, Hanke, & Mann, 2007; Zhang & Shah, 2007), where the Orbitrap can facilitate determination of the charge states of fragment ions, and identification of PTMs by database searching.
- This hybrid mass spectrometer have also been used in environmental chemistry (Krauss & Hollender, 2008), drug analysis (Cuyckens et al., 2008), as well as lipidomics (Davis et al., 2008).

In this thesis, employing sample fractionation and differential gel electrophoresis, we analyzed gel slices by Velos Orbitrap MS. This approach yielded various schistosome antigens as well as many differentially-expressed host proteins in the serum of acute and chronic infected mice.

Relative quantitation:

Proteomics has been defined by Anderson and Anderson (1998) as “the use of quantitative protein-level measurements of gene expression to characterize biological processes

(e.g., disease processes and drug effects) and decipher the mechanisms of gene expression control." Quantitative proteomics is the attempt to quantitate specific proteins found during discovery screening (Ahrens et al., 2010). Quantification is essential for proteomics research since it plays a critical role in biomarker discovery. Proteomic profiling, which compares two or multiple samples of biological relevance, has become a principle strategy to identify biomarkers. There are two main strategies for quantitative MS-based proteomics: stable isotope-based labeling and label-free quantitative methods.

Stable Isotope Labeling

In these techniques, peptides are tagged with different stable isotopes such as ^{2}H, ^{15}N, ^{13}C that facilitate the unbiased comparison of protein amounts in different samples in a single mass spectrometric experiment. The isotope label used is, and except for its mass, similar to the analyzed peptide in physical and chemical properties. Depending on the type of labelling used, whether metabolic or chemical, labels are added in different ways to the samples. When detecting for variables in the same sample sets, metabolic and chemical labels are commonly used because they have a high sensitivity. However, during proteolysis, the release of the selected proteotypic peptide can vary and the quantification derived from the reference peptide is not absolute. In fact, the quantification is absolute only when tryptic fragmentation is complete without a single missed cleavage (Meyer, 2014). Unfortunately, adding isotope labels to the sample is costly, time-consuming, and decreases the sensitivity of the detection. Additionally, the number of isotope labels available is limited (Domon & Aebersold, 2006; Mueller et al., 2008).

• ***In vivo*** **metabolic labeling**: In this method, the label is added to the organism or whole cell during growth (Ong et al., 2002), for example, this can be done by adding stable isotope-containing amino acids to the cell culture media. This method is called stable isotope labeling by

amino acids in cell culture (SILAC). SILAC has been used to study different aspects of mammalian signalling biology, protein-protein interactions (Blagoev et al., 2003; Schulze & Mann, 2004; Selbach & Mann, 2006), and protein dynamics (Blagoev, 2004; Olsen et al. 2006).

• ***In vitro*** **chemical labeling**: In this method, labels are added to proteins or tryptic peptides through a chemical reaction. This method is used to study the oxidation or reduction status of proteins. The most commonly used chemical isotope label is the isotope-coded affinity tag (ICAT) which binds to sulfhydryl groups of cysteine residues (Gygi et al., 1999).

Label-free

Label-free quantitative methods require spectral counting or the measurement of precursor ion intensity. These methods are based on chromatographic data, MS spectra and MS/MS-based peptide identification. Due to their simplicity, affordability and flexibility, label-free quantitative strategies are the method of choice in quantitative LC/MS/MS-based proteomics (Gao et al., 2008; Ramus et al., 2016). Washburn & Yates (2001) used comparative methods such as sequence coverage and peptide number to show that relative protein abundance is correlated with the most with spectral counts. Spectral counting has a large dynamic range (Zybailov et al., 2005; Asara et al., 2008), which allows abundance differences of up to 60:1 to be detected. On the other hand, in SILAC experiments, the detectable ratio is only 20:1. When a mass analyzer with a high MS/MS sampling rate (such as a linear ion trap) is used, the sensitivity of spectral counting increases. Additionally, the accuracy of quantification by ion intensities can be improved using a high resolution mass analyzer such as the Orbitrap. In our studies, we combined spectral counting, amino acid coverage rate, protein identification probability and the number of identified peptides as a label-free method for protein quantification.

Proteomics and Schistosomiasis:

The proteome is defined as the characterization of the total proteins in a certain tissue (e.g.: serum, blood) or cell at a given time. The recent sequencing of the three key schistosome species genomes has enabled the discovery of several new possible vaccine and drug targets, using sensitive and high-throughput proteomics method (Driguez et al., 2015). However, the causes of host pathology caused by schistosomiasis are still not completely clear. To date, several different proteomics techniques have been used in schistosomiasis studies to detect and identify schistosomal proteins.

1- Comparison of soluble schistosomal proteins in different life cycle stages:

Using MALDI- TOF-MS, Curwen et al., 2004 studied *S. mansoni* soluble proteins from the four different life stages, cercariae, schistosomula, adult and eggs *in virto*. This study selected the 40 most dominant soluble proteins and identified only a small number of differences in the composition of the proteins between each life cycle stage (Curwen et al., 2004). Pereira and colleagues were able to identify many putative ubiquitin-specific proteases in *Schistosoma mansoni*. They reported stage-specific expression profiles all through the parasite life cycle that might indicate the use of these proteases in the parasite development (Pereira et al., 2015).

2- Comparison between male and female protein compositions:

Cheng et al., 2005 investigated the protein composition of male and female adults of *S. japonicum*. Using a combination of MALDI-TOF-MS and LC-MS/MS, these authors studied soluble and membrane proteins and detected 52 specific and 49 specific protein for male and female adult worms respectively. However, only 16 and 12 proteins respectively were subsequently identified. Most of the identified proteins were classified as involved in signal transduction and transcriptional regulation, while a small proportion were classified as important for reproduction (Cheng et al., 2005).

3- Proteins of the adult and schistosomula tegumental outer membrane

Van Hellemond et al., 2007 reported that many proteomic studies have investigated the tegument of the adult worm; this membrane is where the interaction between the worm and the host occurs. The tegument consists of two lipid layers, the outer layer has many properties that are different of plasma membrane. It is divided into two parts, one that sheds rapidly within 11 hour, and the other that sheds very slowly. The inner layer is considered as a true plasma membrane (McLaren and Hockley, 1977; Thompson and Geary, 2003). Braschi and colleagues (2006) studied the differences between tegumental membrane proteins from cytosolic and tegumental membrane associated proteins using different techniques with 2D gel electrophoresis. Because they found that proteins are easily removed by washes, they concluded that many of the proteins in the tegumental fraction are loosely attached to the membranes. (Braschi et al., 2006). Braschi & Wilson, (2006) used biotinylation to identify the proteins exposed on the surface of the tegumental membrane and they identified 24 proteins. In a recent study, a large number of differentially expressed proteins of the tegument from the schistosomula stage of *S. mansoni* were identified using quantitative mass spectrometry methods (Sotillo et al., 2015).

4- Antigens and secreted proteins of the schistosome

To date, several proteomic studies on cercarial and adult secretions have been performed and have shown that the secretory products of *S. mansoni* cercariae include several proteases and many potential immuno-modulators either by one-dimensional SDS-PAGE or two-dimensional electrophoresis (Knudsen et al., 2005) and (Curwen et al., 2006) respectively.

5- The schistosome effect on the protein composition of the host liver

Schistosomiasis causes severe inflammation of the liver. A recent study investigated the effect of schistosome infection on protein composition of the liver in infected mice compared to

non-infected mice. Harvie and colleagues used 2D dimensional gel-electrophoresis and then identified the protein spots by MALDI-MS. In the infected livers, they noticed a marked decrease in proteins involved in diverse liver function such as the urea cycle, fatty acid oxidation and the Krebs cycle, while proteins involved in stress responses and acute phase reactants were increased (Harvie et al., 2007).

Objectives of this Study

I carried out a proteomic profile analysis of human sera taken from patients with schistosomiasis to identify new putative biomarkers. We carried out parallel studies on the sera of mice infected with *S. mansoni* to study the early stage of the disease and to compare biomarker profiles in chronically-infected mice with the chronic stage in humans. This work employed high throughput surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF MS), high precision matrix-assisted laser desorption and ionization (MALDI-TOF MS) mass spectrometry, Electrospray ionization (ESI) as sources for mass spectrometry, and up-to-date mass analyzers, for example Orbitrap.

Research Hypothesis and Aims:

Hypothesis#1: Patients chronically infected with *S. mansoni* have characteristic serum protein profiles.

Hypothesis#2: Infection of mice with *S. mansoni* leads to an alteration of the serum protein profile that change over time.

Aims

The current thesis aims to identify putative biomarkers for schistosomiasis to address gaps in the diagnosis of human schistosomiasis. For this purpose, mass spectrometry (MS)-based biomarker discovery was applied to exploit the capabilities of mass spectrometry for protein

identification and quantification for fast, systematic biomarker discovery. A further aim of this thesis was to compare biomarkers in mouse and human schistosomiasis.

References

Abbasi, I., King, C. H., Muchiri, E. M., & Hamburger, J. (2010). Detection of Schistosoma mansoni and Schistosoma haematobium DNA by loop-mediated isothermal amplification: identification of infected snails from early prepatency. *The American Journal of Tropical Medicine and Hygiene*, *83*(2), 427-432.

Ahrens, C. H., Brunner, E., Qeli, E., Basler, K., & Aebersold, R. (2010). Generating and navigating proteome maps using mass spectrometry. *Nature Reviews Molecular Cell Biology, 11*(11), 789-801.

Amiri, P., Haak-Frendscho, M., Robbins, K., McKerrow, J. H., Stewart, T., & Jardieu, P. (1994). Anti-immunoglobulin E treatment decreases worm burden and egg production in Schistosoma mansoni-infected normal and interferon gamma knockout mice. *The Journal of Experimental Medicine, 180*(1), 43-51.

Anderson, N. L., & Anderson, N. G. (1998). Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis, 19*(11), 1853-1861.

Asahi, H., & Stadecker, M. J. (2003). Analysis of egg antigens inducing hepatic lesions in schistosome infection. *Parasitology International, 52*(4), 361-367.

Ashton, P. D., Harrop, R., Shah, B., & Wilson, R. A. (2001). The schistosome egg: development and secretions. *Parasitology, 122*(03), 329-338.

Asara, J. M., Christofk, H. R., Freimark, L. M., & Cantley, L. C. (2008). A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen. *Proteomics, 8*(5), 994-999.

Atanassov, C., Bonifait, L., Perivier, M., Gottschalk, M., & Grenier, D. (2015). Candidate proteomic biomarkers for three genogroups of the swine pathogen Streptococcus suis serotype 2. *BMC Microbiology, 15*(1), 84.

Barreto, V.S.T. (1971). Alkaline phosphatase in schistosomiasis. *Annals of Internal Medicine, 74*(3), 450-451.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell, 116*(2), 281-297.

Barylyuk, K., Gülbakan, B., Xie, X., & Zenobi, R. (2013). DNA Oligonucleotides: A model system with tunable binding strength to study monomer–dimer equilibria with electrospray ionization-mass spectrometry. *Analytical Chemistry, 85*(24), 11902-11912.

Basch, P. F. (1981). Cultivation of Schistosoma mansoni in vitro. I. Establishment of cultures from

cercariae and development until pairing. *The Journal of Parasitology*, 179-185.

Bensimon, A., Heck, A. J., & Aebersold, R. (2012). Mass spectrometry-based proteomics and network biology. *Annual Review of Biochemistry, 81*, 379-405.

Bergquist, R., Al-Sherbiny, M., Barakat, R., & Olds, R. (2002). Blueprint for schistosomiasis vaccine development. *Acta Tropica, 82*(2), 183-192.

Bergquist, N. R., Leonardo, L. R., & Mitchell, G. F. (2005). Vaccine-linked chemotherapy: can schistosomiasis control benefit from an integrated approach?. *Trends in Parasitology, 21*(3), 112-117.

Bergquist, R., UTZINGER, J., Chollet, J., Shu-Hua, X., Weiss, N. A., & Tanner, M. (2004). Triggering of high-level resistance against Schistosoma mansoni reinfection by artemether in the mouse model. *The American Journal of Tropical Medicine and Hygiene, 71(6), 774-777.*

Bhatt, A., Parsi, M. A., Stevens, T., Gabbard, S., Kumaravel, A., Jang, S.& Dweik, R. (2015). Volatile Organic Compounds in Plasma for the Diagnosis of Esophageal Adenocarcinoma: A Pilot Study. Gastrointestinal Endoscopy.

Downing, G. (2001). Biomarkers Definitions Working Group. Biomarkers and Surrogate Endpoints. *Clinical Pharmacology & Therapeutics, 69*(3), 89-95.

Bickle, Q. D., Bøgh, H. O., Johansen, M. V., & Zhang, Y. (2001). Comparison of the vaccine efficacy of γ-irradiated Schistosoma japonicum cercariae with the defined antigen Sj62 (IrV-5) in pigs. *Veterinary Parasitology, 100*(1), 51-62.

Blagoev, B., Kratchmarova, I., Ong, S. E., Nielsen, M., Foster, L. J., & Mann, M. (2003). A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nature Biotechnology, 21*(3), 315-318.

Blagoev, B., Ong, S. E., Kratchmarova, I., & Mann, M. (2004). Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. *Nature Biotechnology, 22*(9), 1139-1145.

Bogitsh, B. J. (1975). Cytochemical observations on the gastrodermis of digenetic trematodes. *Transactions of the American Microscopical Society, 94*(4), 524-528.

Bogitsh, B. J. (1982). Schistosoma mansoni: cytochemistry and morphology of the gastrodermal

Golgi apparatus. *Experimental parasitology, 53*(1), 57-67.

Borojevic, R. (1992). Experimental murine schistosomiasis mansoni: estabilishment of the chronic phase of the disease. *Memórias do Instituto Oswaldo Cruz*, *87*(4), 171-174.

Boros, D. L. (1999). T helper cell populations, cytokine dynamics, and pathology of the schistosome egg granuloma. *Microbes and Infection, 1*(7), 511-516.

Boros, D. L., & Whitfield, J. R. (1998). Endogenous IL-10 regulates IFN-gamma and IL-5 cytokine production and the granulomatous response in Schistosomiasis mansoni-infected mice. *Immunology, 94*(4), 481.

Braschi, S., Borges, W. C., & Wilson, R. A. (2006). Proteomic analysis of the shistosome tegument and its surface membranes. *Memorias do Instituto Oswaldo Cruz, 101*(1), 205-212.

Braschi, S., & Wilson, R. A. (2006). Proteins exposed at the adult schistosome surface revealed by biotinylation. *Molecular & Cellular Proteomics, 5*(2), 347-356.

Buchan, B. W., & Ledeboer, N. A. (2014). Emerging technologies for the clinical microbiology laboratory. *Clinical Microbiology Reviews, 27*(4), 783-822.

Butler-Wu, S. M., Burns, E. M., Pottinger, P. S., Magaret, A. S., Rakeman, J. L., Matsen, F. A., &

Cookson, B. T. (2011). Optimization of periprosthetic culture for diagnosis of Propionibacterium acnes prosthetic joint infection. *Journal of Clinical Microbiology, 49*(7), 2490-2495.

Cai, P., Hou, N., Piao, X., Liu, S., Liu, H., Yang, F. & Chen, Q. (2011). Profiles of small non-coding RNAs in Schistosoma japonicum during development. *PLoS Negl Trop Dis, 5*(8), e1256.

Caldas, I. R., Campi-Azevedo, A. C., Oliveira, L. F. A., Silveira, A. M. S., Oliveira, R. C., & Gazzinelli, G. (2008). Human schistosomiasis mansoni: immune responses during acute and chronic phases of the infection. *Acta Tropica, 108*(2), 109-117.

Cardoso, F. C., Pinho, J. M. R., Azevedo, V., & Oliveira, S. C. (2006). Identification of a new Schistosoma mansoni membrane-bound protein through bioinformatic analysis. *Genet Mol Res, 5*(4), 609-618.

Chapman, K. (2002). The ProteinChip (R) Biomarker System from Ciphergen Biosystems: a

novel proteomics platform for rapid biomarker discovery and validation. *Biochemical Society Transactions 30*(2), 82-7.

Chen, X., Liang, H., Zhang, J., Zen, K., & Zhang, C. Y. (2012). Secreted microRNAs: a new form

of intercellular communication. *Trends in Cell Biology, 22*(3), 125-132.

Cheng, G. F., Lin, J. J., Feng, X. G., Fu, Z. Q., Jin, Y. M., Yuan, C. X., ... & Cai, Y. M. (2005). Proteomic analysis of differentially expressed proteins between the male and female worm of Schistosoma japonicum after pairing. *Proteomics, 5*(2), 511-521.

Cheng, G., Luo, R., Hu, C., Cao, J., & Jin, Y. (2013). Deep sequencing-based identification of pathogen-specific microRNAs in the plasma of rabbits infected with Schistosoma japonicum. *Parasitology, 140*(14), 1751-1761.

Clarke, W., Silverman, B. C., Zhang, Z., Chan, D. W., Klein, A. S., & Molmenti, E. P. (2003). Characterization of renal allograft rejection by urinary proteomic analysis. *Annals of Surgery, 237*(5), 660-665.

Cockburn, A., Barraco, R. A., Reyman, T. A., & Peck, W. (1975). Autopsy of an Egyptian mummy. *Science, 187*(4152), 1155-1160.

Colley, D. G., & Secor, W. E. (2014). Immunology of human schistosomiasis. *Parasite Immunology, 36*(8), 347-357.

Comin, F., Speziali, E., Correa-Oliveira, R., & Faria, A. M. C. (2008). Aging and immune response

in chronic human schistosomiasis. *Acta Tropica, 108*(2), 124-130.

Contis, G., & David, A. R. (1996). The epidemiology of bilharzia in ancient Egypt: 5000 years of schistosomiasis. *Parasitology Today, 12*(7), 253-255.

Corrêa-Oliveira, R., Caldas, I. R., & Gazzinelli, G. (2000). Natural versus drug-induced resistance

in Schistosoma mansoni infection. *Parasitology Today, 16*(9), 397-399.

Correa-Oliveira, R., Pearce, E. J., Oliveira, G. C., Golgher, D. B., Katz, N., Bahia, L. G., ... & Sher, A. (1989). The human immune response to defined immunogens of Schistosoma mansoni: elevated antibody levels to paramyosin in stool-negative individuals from two endemic areas in Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 83*(6), 798-804.

Corstjens, P. L., van Lieshout, L., Zuiderwijk, M., Kornelis, D., Tanke, H. J., Deelder, A. M., & van Dam, G. J. (2008). Up-converting phosphor technology-based lateral flow assay for detection of Schistosoma circulating anodic antigen in serum. *Journal of Clinical Microbiology, 46*(1), 171-176.

Cousin, C. E., & Dorsey, C. H. (1991). Nervous system ofSchistosoma mansoni cercaria: Organization and fine structure. *Parasitology Research, 77*(2), 132-141.

Cox, T. R., Schoof, E. M., Gartland, A., Erler, J. T., & Linding, R. (2015). Dataset for the proteomic inventory and quantitative analysis of the breast cancer hypoxic secretome associated with osteotropism. *Data in Brief*, 5, 621-625.

Crabtree, J. E., & Wilson, R. A. (1985). Schistosoma mansoni: an ultrastructural examination of skin migration in the hamster cheek pouch. *Parasitology, 91*(01), 111-120.

Crabtree, J. E., & Wilson, R. A. (1980). Schistosoma mansoni: a scanning electron microscope study of the developing schistosomulum. *Parasitology, 81*(03), 553-564.

Croxatto, A., Prod'hom, G., & Greub, G. (2012). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiology Reviews, 36*(2), 380-407.

Cui, J., Kang, X., Dai, Z., Huang, C., Zhou, H., Guo, K., ... & Liu, Y. (2007). Prediction of chronic hepatitis B, liver cirrhosis and hepatocellular carcinoma by SELDI-based serum decision tree classification. *Journal of Cancer Research and Clinical Oncology, 133*(11), 825-834.

Cunningham Jr, C., Glish, G. L., & Burinsky, D. J. (2006). High amplitude short time excitation: a method to form and detect low mass product ions in a quadrupole ion trap mass spectrometer. *Journal of the American Society for Mass Spectrometry, 17*(1), 81-84.

Curwen, R. S., Ashton, P. D., Johnston, D. A., & Wilson, R. A. (2004). The Schistosoma mansoni soluble proteome: a comparison across four life-cycle stages. *Molecular and Biochemical Parasitology, 138*(1), 57-66.

Curwen, R. S., Ashton, P. D., Sundaralingam, S., & Wilson, R. A. (2006). Identification of novel proteases and immunomodulators in the secretions of schistosome cercariae that facilitate host entry. *Molecular & Cellular Proteomics, 5*(5), 835-844.

Cuyckens, F., Balcaen, L. I., De Wolf, K., De Samber, B., Van Looveren, C., Hurkmans, R., &

Vanhaecke, F. (2008). Use of the bromine isotope ratio in HPLC-ICP-MS and HPLC-ESI-MS analysis of a new drug in development. *Analytical and Bioanalytical Chemistry, 390*(7), 1717-1729.

Davis, A. (2009). Schistosomiasis, in: G.C. Cook, A.I. Zumba (Eds.), Manson's tropical diseases .*WB Saunders, 2003* (21), 1425–1460.

Davis, B., Koster, G., Douet, L. J., Scigelova, M., Woffendin, G., Ward, J. M. & Postle, A. D. (2008). Electrospray ionization mass spectrometry identifies substrates and products of lipoprotein-associated phospholipase A2 in oxidized human low density lipoprotein. *Journal of Biological Chemistry, 283*(10), 6428-6437.

De Gruttola, V. G., Clax, P., DeMets, D. L., Downing, G. J., Ellenberg, S. S., Friedman, L., ... & Zeger, S. L. (2001). Considerations in the evaluation of surrogate endpoints in clinical trials: summary of a National Institutes of Health workshop. *Controlled Clinical Trials, 22*(5), 485-502.

Deelder, A. M., De Jonge, N., Boerman, O. C., Fillié, Y. E., Hilberath, G. W., Rotmans, J. P., & Schut, D. W. (1989). Sensitive determination of circulating anodic antigen in Schistosoma mansoni infected individuals by an enzyme-linked immunosorbent assay using monoclonal antibodies. *The American Journal of Tropical Medicine and Hygiene, 40*(3), 268-272.

Deelder, A. M., Klappe, H. T. M., Van den Aardweg, G. J. M. J., & Van Meerbeke, E. H. E. M. (1976). Schistosoma mansoni: Demonstration of two circulating antigens in infected hamsters. *Experimental Parasitology, 40*(2), 189-197.

Deelder, A. M., Kornelis, D., Van Marck, E. A. E., Eveleigh, P. C., & Van Egmond, J. G. (1980). Schistosoma mansoni: Characterization of two circulating polysaccharide antigens and the immunological response to these antigens in mouse, hamster, and human infections. *Experimental parasitology, 50*(1), 16-32.

De Noya, B. A., Ruiz, R., Losada, S., Colmenares, C., Contreras, R., Cesari, I. M., & Noya, O. (2007). Detection of schistosomiasis cases in low-transmission areas based on coprologic and serologic criteria: the Venezuelan experience. *Acta tropica, 103*(1), 41-49.

De Souza Gomes, M., Muniyappa, M. K., Carvalho, S. G., Guerra-Sá, R., & Spillane, C. (2011). Genome-wide identification of novel microRNAs and their target genes in the human parasite Schistosoma mansoni. Genomics, 98(2), 96-111.

Diamandis, E. P. (2004). Mass spectrometry as a diagnostic and a cancer biomarker discovery tool opportunities and potential limitations. *Molecular & Cellular Proteomics, 3*(4), 367-378.

Dimmette, R. M. (1955). Liver biopsy in clinical schistosomiasis; comparison of wedge and needle types. *Gastroenterology, 29*(2), 219-234.

Dieckmann, R., Helmuth, R., Erhard, M., & Malorny, B. (2008). Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology, 74*(24), 7767-7778.

Doenhoff, M. J., Chiodini, P. L., & Hamilton, J. V. (2004). Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies?. *Trends in Parasitology*, *20*(1), 35-39.

Domingues, A. L. C., Lima, A. R. F., Dias, H. S., Leao, G. C., & Coutinho, A. (1993). An ultrasonographic study of liver fibrosis in patients infected with Schistosoma mansoni in north-east Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 87*(5), 555-558.

Domingues, A. L. C., Medeiros, T. B. D., & Lopes, E. P. D. A. (2011). Ultrasound versus biological markers in the evaluation of periportal fibrosis in human Schistosoma mansoni. *Memórias do Instituto Oswaldo Cruz, 106*(7), 802-807.

Domon, B., & Aebersold, R. (2006). Mass spectrometry and protein analysis. *Science, 312*(5771), 212-217.

Dorsey, C. H. (1975). Schistosoma mansoni: Development of acetabular glands of cercaria at ultrastructural level. *Experimental Parasitology, 37*(1), 37-59.

Dorsey, C. H. (1976). Schistosoma mansoni: description of the head gland of cercariae and schistosomules at the ultrastructural level. *Experimental Parasitology, 39*(3), 444-459.

Dos Santos, V. N., Leite-Mór, M. M. B., Kondo, M., Martins, J. R., Nader, H., Lanzoni, V. P., & Parise, E. R. (2005). Serum laminin, type IV collagen and hyaluronan as fibrosis markers in non-alcoholic fatty liver disease. *Brazilian Journal of Medical and Biological Research, 38*(5), 747-753.

Driguez, P., McManus, D. P., & Gobert, G. N. (2015). Clinical implications of recent findings in schistosome proteomics. *Expert Review of Proteomics*, *13*(1), 19-33.

Ebrahimzadeh, A., & Kraft, M. (1969). Ultrastructural studies on the morphology of the cercariae

Schistosoma mansoni. I. The alimentary tract. *Zeitschrift für Parasitenkunde (Berlin, Germany), 32*(2), 157.

Eliuk, S., & Makarov, A. A. (2015). Evolution of Orbitrap Mass Spectrometry Instrumentation. *Annual Review of Analytical Chemistry, 8*(1), 61-80.

Engwegen, J. Y., Gast, M. C. W., Schellens, J. H., & Beijnen, J. H. (2006). Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. *Trends in Pharmacological Sciences, 27*(5), 251-259.

Enk, M. J., Amaral, G. L., Silveira-Lemos, D., Teixeira-Carvalho, A., Martins-Filho, O. A., Correa-Oliveira, R., & Massara, C. L. (2010). Rural tourism: a risk factor for schistosomiasis transmission in Brazil. *Memórias do Instituto Oswaldo Cruz, 105*(4), 537-540.

Espy, M. J., Uhl, J. R., Sloan, L. M., Buckwalter, S. P., Jones, M. F., Vetter, E. A., ... & Smith, T. F. (2006). Real-time PCR in clinical microbiology: *applications for routine laboratory testing. Clinical Microbiology Reviews, 19*(1), 165-256.

Evan Secor, W. (2014). Water-based interventions for schistosomiasis control. *Pathogens and Global Health, 108*(5), 246-254.

Fabris, D. (2011). MS analysis of nucleic acids in the post-genomic era. *Analytical Chemistry, 83*(15), 5810-5816.

Fenollar, F., Gonçalves, A., Esterni, B., Azza, S., Habib, G., Borg, J. P., & Raoult, D. (2006). A serum protein signature with high diagnostic value in bacterial endocarditis: results from a study based on surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. *Journal of Infectious Diseases, 194*(10), 1356-1366.

Fenn, J. B. (2003). Electrospray wings for molecular elephants (Nobel lecture). *Angewandte Chemie International Edition, 42*(33), 3871-3894.

Fenselau, C., & Demirev, P. A. (2001). Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrometry Reviews, 20*(4), 157-171.

Foo, K. T., Blackstock, A. J., Ochola, E. A., Matete, D. O., Mwinzi, P. N., Montgomery, S. P., ... & Secor, W. E. (2015). Evaluation of Point-of-Contact Circulating Cathodic Antigen Assays for the Detection of Schistosoma mansoni Infection in Low-, Moderate-, and

High-Prevalence Schools in Western Kenya. *The American Journal of Tropical Medicine and Hygiene, 92*(6), 1227-1232.

Freeman, C. M., Stolberg, V. R., Chiu, B. C., Lukacs, N. W., Kunkel, S. L., & Chensue, S. W. (2006). CCR4 participation in Th type 1 (mycobacterial) and Th type 2 (schistosomal) anamnestic pulmonary granulomatous responses. *The Journal of Immunology, 177*(6), 4149-4158.

Gabelica, V., Vreuls, C., Filée, P., Duval, V., Joris, B., & Pauw, E. D. (2002). Advantages and drawbacks of nanospray for studying noncovalent protein–DNA complexes by mass spectrometry. *Rapid Communications in Mass Spectrometry, 16*(18), 1723-1728.

Gallo, c., ciavarella, d., santarelli, a., ranieri, e., colella, g., muzio, l. L., & russo, l. L. (2016). Potential Salivary Proteomic Markers of Oral Squamous Cell Carcinoma. *Cancer Genomics-Proteomics, 13*(1), 55-61.

Gao, B. B., Stuart, L., & Feener, E. P. (2008). Label-free Quantitative Analysis of One-dimensional PAGE LC/MS/MS Proteome Application on Angiotensin II-Stimulated Smooth Muscle Cells Secretome. *Molecular & Cellular Proteomics, 7*(12), 2399-2409.

Gerosa, F., Baldani-Guerra, B., Nisii, C., Marchesini, V., Carra, G., & Trinchieri, G. (2002). Reciprocal activating interaction between natural killer cells and dendritic cells. *The Journal of Experimental Medicine, 195*(3), 327-333.

Gidden, J., Ferzoco, A., Baker, E. S., & Bowers, M. T. (2004). Duplex formation and the onset of

helicity in poly d (CG) n oligonucleotides in a solvent-free environment. *Journal of the American Chemical Society, 126*(46), 15132-15140.

Giebel, R., Worden, C., Rust, S. M., Kleinheinz, G. T., Robbins, M., & Sandrin, T. R. (2010). Microbial fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS): applications and challenges. *Advances in Applied Microbiology, 71*(1), 149-184.

Gray, D. J., Ross, A. G., Li, Y. S., & McManus, D. P. (2011). Diagnosis and management of schistosomiasis. *BMJ, 342*(2), 2651-2651.

Grenfell, R. F. Q., Silva-Moraes, V., Taboada, D., de Mattos, A. C. A., de Castro, A. K. S., & Coelho, P. M. Z. (2012). Immunodiagnostic methods: what is their role in areas of low endemicity? *The Scientific World Journal*, *2012*.

Grogan, J. L., Kremsner, P. G., van Dam, G. J., Metzger, W., Mordmüller, B., Deelder, A. M., & Yazdanbakhsh, M. (1996). Antischistosome IgG4 and IgE responses are affected differentially by chemotherapy in children versus adults. *Journal of Infectious Diseases,* 173(5), 1242-1247.

Gryseels, B., Polman, K., Clerinx, J., & Kestens, L. (2006). Human schistosomiasis. *The Lancet, 368*(9541), 1106-1118.

Gryseels, B. (2012). Schistosomiasis. *Infect Dis Clin North Am, 26*(2):383-97.

Gstaiger, M., & Aebersold, R. (2009). Applying mass spectrometry-based proteomics to genetics, genomics and network biology. *Nature Reviews Genetics, 10*(9), 617-627.

Guilhaus, M., Mlynski, V., & Selby, D. (1997). Perfect Timing: Time-of-flight Mass Spectrometry. *Rapid Communications in Mass Spectrometry, 11*(9), 951-962.

Guo, Y., Shibuya, K., Cheng, G., Rao, K., Lee, L., & Tang, S. (2010). Tracking China's health reform. *The Lancet, 375*(9720), 1056-1058.

Guo, Z., Zhang, Q., Zou, H., Guo, B., and Ni, J. (2002). A method for the analysis of low-mass molecules by MALDI-TOF mass spectrometry. *Analytical Chemistry, 74*(7), 1637-1641.

Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., & Aebersold, R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature Biotechnology, 17*(10), 994-999.

Hagan, P., Blumenthal, U. J., Dunn, D., Simpson, A. J., & Wilkins, H. A. (1991). Human IgE, IgG4 and resistance to reinfection with Schistosoma haematobium. *Nature, 349,* 243-245.

Hagen, J., Scheerlinck, J. P. Y., Young, N. D., Gasser, R. B., & Kalinna, B. H. (2015). Chapter Three-Prospects for Vector-Based Gene Silencing to Explore Immunobiological Features of Schistosoma mansoni. *Advances in Parasitology, 88*(30), 85-122.

Hager, J. W. (2002). A new linear ion trap mass spectrometer. *Rapid Communications in Mass Spectrometry, 16*(6), 512-526.

Hadi, N. I., & Jamal, Q. (2015). "OMIC" tumor markers for breast cancer: A review. *Pak J Med Sci. 31*(5): 1256–1262.

Harvie, M., Jordan, T. W., & La Flamme, A. C. (2007). Differential liver protein expression during schistosomiasis. *Infection and immunity, 75*(2), 736-744.

He, X., Sai, X., Chen, C., Zhang, Y., Xu, X., Zhang, D., & Pan, W. (2013). Host serum miR-223

is a potential new biomarker for Schistosoma japonicum infection and the response to chemotherapy. *Parasit Vectors*, 6, 272-279.

Helfer, A. G., Michely, J. A., Weber, A. A., Meyer, M. R., & Maurer, H. H. (2015). Orbitrap technology for comprehensive metabolite-based liquid chromatographic–high resolution-tandem mass spectrometric urine drug screening–Exemplified for cardiovascular drugs. *Analytica Chimica Acta*, 891, 221-233.

Henri, S., Chevillard, C., Mergani, A., Paris, P., Gaudart, J., Camilla, C., ... & Dessein, A. J. (2002).

Cytokine regulation of periportal fibrosis in humans infected with Schistosoma mansoni: IFN-γ is associated with protection against fibrosis and TNF-α with aggravation of disease. *The Journal of Immunology, 169*(2), 929-936.

Henderson, G. S., Lu, X., McCurley, T. L., & Colley, D. G. (1992). In vivo molecular analysis of lymphokines involved in the murine immune response during Schistosoma mansoni infection. II. Quantification of IL-4 mRNA, IFN-gamma mRNA, and IL-2 mRNA levels in the granulomatous livers, mesenteric lymph nodes, and spleens during the course of modulation. *The Journal of Immunology, 148*(7), 2261-2269.

Herrero, M., Simó, C., García-Cañas, V., Ibáñez, E., & Cifuentes, A. (2012). Foodomics: MS-based strategies in modern food science and nutrition. *Mass Spectrometry Reviews, 31*(1), 49-69.

Hockley, D. J. (1972). Schistosoma mansoni: the development of the cercarial tegument. *Parasitology, 64*(02), 245-252.

Holland, R. D., Duffy, C. R., Rafii, F., Sutherland, J. B., Heinze, T. M., Holder, C. L. & Lay Jr, J. O. (1999). Identification of bacterial proteins observed in MALDI-TOF mass spectra from whole cells. *Analytical Chemistry, 71*(15), 3226-3230.

Hong, Y., Zhang, M., Yang, J., Cao, X., Han, Q., Han, Y. & Fu, Z. (2015). Immunoproteomic analysis of Schistosoma japonicum schistosomulum proteins recognized by immunoglobulin G in the sera of susceptible and non-susceptible hosts. *Journal of Proteomics, 124,* 25-38.

Hopper, J. T., & Robinson, C. V. (2014). Mass Spectrometry Quantifies Protein Interactions—From Molecular Chaperones to Membrane Porins. *Angewandte Chemie International Edition, 53*(51), 14002-14015.

Horton, M. A., Larson, K. A., Lee, J. J., & Lee, N. A. (1996). Cloning of the murine eosinophil peroxidase gene (mEPO): characterization of a conserved subgroup of mammalian hematopoietic peroxidases. *Journal of Leukocyte Biology, 60*(2), 285-294.

Hoy, A. M., Lundie, R. J., Ivens, A., Quintana, J. F., Nausch, N., Forster, T. & Buck, A. H. (2014). Parasite-derived microRNAs in host serum as novel biomarkers of helminth infection. *PLoS Negl Trop Dis, 8*(2), e2701.

Hu, Q., Noll, R. J., Li, H., Makarov, A., Hardman, M., & Graham Cooks, R. (2005). The Orbitrap:

a new mass spectrometer. *Journal of Mass Spectrometry, 40*(4), 430-443.

International Human Genome Sequencing Consortium. (2004). Finishing the euchromatic sequence of the human genome. *Nature, 431*(7011), 931-945.

Iskandar, H. N., & Ciorba, M. A. (2012). Biomarkers in inflammatory bowel disease: current practices and recent advances. *Translational Research, 159*(4), 313-325.

Jacovides, C. L., Kreft, R., Adeli, B., Hozack, B., Ehrlich, G. D., & Parvizi, J. (2012). Successful identification of pathogens by polymerase chain reaction (PCR)-based electron spray ionization time-of-flight mass spectrometry (ESI-TOF-MS) in culture-negative periprosthetic joint infection. *The Journal of Bone & Joint Surgery, 94*(24), 2247-2254.

Jenkins, S. J., Hewitson, J. P., Jenkins, G. R., & Mountford, A. P. (2005). Modulation of the host's

immune response by schistosome larvae. *Parasite Immunology, 27*(10-11), 385-393.

Jones, M. B., Krutzsch, H., Shu, H., Zhao, Y., Liotta, L. A., Kohn, E. C., & Petricoin, E. F. (2002). Proteomic analysis and identification of new biomarkers and therapeutic targets for invasive ovarian cancer. *Proteomics, 2*(1), 76-84.

Jones ME, Mitchell RG, Leen CL, 1992. Long seronegative window in schistosoma infection. *The Lancet, 340*(8834), 1549-1550.

Kang, M. J., Pyun, J. C., Lee, J. C., Choi, Y. J., Park, J. H., Park, J. G., ... & Choi, H. J. (2005). Nanowire-assisted laser desorption and ionization mass spectrometry for quantitative analysis of small molecules. *Rapid Communications in Mass Spectrometry, 19*(21), 3166-3170.

Kang, X., Xu, Y., Wu, X., Liang, Y., Wang, C., Guo, J., ... & Tang, H. (2005). Proteomic

fingerprints for potential application to early diagnosis of severe acute respiratory syndrome. *Clinical Chemistry, 51*(1), 56-64.

Karpova, M. A., Moshkovskii, S. A., Toropygin, I. Y., & Archakov, A. I. (2010). Cancer-specific MALDI-TOF profiles of blood serum and plasma: biological meaning and perspectives. *Journal of Proteomics, 73*(3), 537-551.

Katz, N., Chaves, A., & Pellegrino, J. (1972). A simple device for quantitative stool thick-smear technique in Schistosomiasis mansoni. *Revista do Instituto de Medicina Tropical de São Paulo, 14*(6), 397.

Katz, N., Coelho, P. M. Z., & Pellegrino, J. (1970). Evaluation of Kato's quantitative method through the recovery of Schistosoma mansoni eggs added to human feces. *Journal of Parasitology, 56*(5), 1032-1033.

Kebarle, P., & Verkerk, U. H. (2009). Electrospray: from ions in solution to ions in the gas phase, what we know now. *Mass Spectrometry Reviews, 28*(6), 898-917.

Keller, B. O. and Li, L. (2000). Discerning matrix-cluster peaks in matrix-assisted laser desorption/ionization time-of-flight mass spectra of dilute peptide mixtures. *J. Am. Soc. Mass Spectrom*. 11, 88–93.

Kim, J.I., Park, J.M., Noh, J.Y., Hwang, S.J., Kang, M.J., Pyun, J.C. (2015). Analysis of benzylpenicillin in milk using MALDI-TOF mass spectrometry with top-down synthesized TiO2 nanowires as the solid matrix. *Chemosphere, 143, 64-70.*

Kim, M. S., Kandasamy, K., Chaerkady, R., & Pandey, A. (2010). Assessment of resolution parameters for CID-based shotgun proteomic experiments on the LTQ-Orbitrap mass spectrometer. *Journal of the American Society for Mass Spectrometry, 21*(9), 1606-1611.

King, C. H., Dickman, K., & Tisch, D. J. (2005). Reassessment of the cost of chronic helmintic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. *The Lancet, 365*(9470), 1561-1569.

Klein, D. (2002). Quantification using real-time PCR technology: applications and limitations. *Trends in molecular medicine, 8*(6), 257-260.

Knochenmuss, R. (2006). Ion formation mechanisms in UV-MALDI. *Analyst, 131*(9), 966-986.

Knudsen, G. M., Medzihradszky, K. F., Lim, K. C., Hansell, E., & McKerrow, J. H. (2005). Proteomic analysis of Schistosoma mansoni cercarial secretions. *Molecular & Cellular Proteomics, 4*(12), 1862-1875.

Krauss, M., & Hollender, J. (2008). Analysis of nitrosamines in wastewater: exploring the trace level quantification capabilities of a hybrid linear ion trap/orbitrap mass spectrometer. *Analytical chemistry, 80*(3), 834-842.

Laughlin, S., & Wilson, W. D. (2015). May the Best Molecule Win: Competition ESI Mass Spectrometry. *International journal of molecular sciences, 16*(10), 24506-24531.

Lawrence, J. D. (1973). The ingestion of red blood cells by Schistosoma mansoni. *The Journal of parasitology*, 60-63.

Lewin, P. K. (1977). Mummies that I have known: A pediatrician's venture in the field of paleopathology. *American Journal of Diseases of Children, 131*(3), 349-350.

Lightowlers, M. W., & Rickard, M. D. (1988). Excretory–secretory products of helminth parasites:

effects on host immune responses. *Parasitology, 96*(1), 123-166.

Light-Wahl, K. J., Springer, D. L., Winger, B. E., Edmonds, C. G., Camp, D. G., Thrall, B. D., & Smith, R. D. (1993). Observation of a small oligonucleotide duplex by electrospray ionization mass spectrometry. *Journal of the American Chemical Society, 115*(2), 803-804.

Lopez, A. D., Mathers, C. D., Ezzati, M., Jamison, D. T., & Murray, C. J. (2006). Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *The Lancet, 367*(9524), 1747-1757.

Loukas, A., Tran, M., & Pearson, M. S. (2007). Schistosome membrane proteins as vaccines. *International journal for parasitology, 37*(3), 257-263.

Madsen, J. A., & Brodbelt, J. S. (2010). Asymmetric charge partitioning upon dissociation of DNA

duplexes. *Journal of the American Society for Mass Spectrometry, 21*(7), 1144-1150.

Maher, S., Syed, S. U., Hughes, D. M., Gibson, J. R., & Taylor, S. (2013). Mapping the stability diagram of a quadrupole mass spectrometer with a static transverse magnetic field applied. *Journal of the American Society for Mass Spectrometry, 24*(8), 1307-1314.

Makarov, A., Denisov, E., Lange, O., & Horning, S. (2006). Dynamic range of mass accuracy in LTQ Orbitrap hybrid mass spectrometer. *Journal of the American Society for Mass Spectrometry, 17*(7), 977-982.

Makarov, A. (2000). Electrostatic axially harmonic orbital trapping: a high-performance

technique of mass analysis. *Analytical chemistry, 72*(6), 1156-1162.

Makioka, A., Kumagai, M., Kobayashi, S., & Takeuchi, T. (2007). Differences in protein profiles of the isolates of Entamoeba histolytica and E. dispar by surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) ProteinChip assays. *Parasitology Research, 102*(1), 103-110.

Mair, G. R., Maule, A. G., Fried, B., Day, T. A., & Halton, D. W. (2009). Organization of the musculature of schistosome cercariae. *Journal of Parasitology, 89*(3), 623-625.

Manivannan, B., Rawson, P., Jordan, T. W., Secor, W. E., & La Flamme, A. C. (2010). Differential patterns of liver proteins in experimental murine hepatosplenic schistosomiasis. *Infection and Immunity, 78*(2), 618-628.

Mansour, M. M., Farid, Z., Bassily, S., Salah, L. H., & Watten, R. H. (1982). Serum enzyme tests in hepatosplenic schistosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 76*(1), 109-111.

March, R. E. (2000). Quadrupole ion trap mass spectrometry: a view at the turn of the century. *International Journal of Mass Spectrometry, 200*(1), 285-312.

Marshall, J., Bowden, P., Schmit, J. C., & Betsou, F. (2014). Creation of a federated database of blood proteins: a powerful new tool for finding and characterizing biomarkers in serum. *Clinical proteomics, 11*(1), 3.

Marshall, A. G., Hendrickson, C. L., & Jackson, G. S. (1998). Fourier transform ion cyclotron resonance mass spectrometry: a primer. *Mass spectrometry reviews, 17*(1), 1-35.

McLaren, D. J. (1984). Disguise as an evasive stratagem of parasitic organisms. *Parasitology 88*, 597–611.

McLaren, D. J., and Terry, R. J. (1982). The protective role of acquired host antigens during schistosome maturation. *Parasite Immunol*. 4, 129–148

McLaren, D. J., & Hockley, D. J. (1976). Schistosoma mansoni: the occurrence of microvilli on the surface of the tegument during transformation from cercaria to schistosomulum. *Parasitology, 73*(02), 169-187.

McManus, D. P., & Loukas, A. (2008). Current status of vaccines for schistosomiasis. *Clinical Microbiology Reviews, 21*(1), 225-242.

Merchant, M., & Weinberger, S. R. (2000). Recent advancements in surface-enhanced laser

desorption/ionization-time of flight-mass spectrometry. *Electrophoresis, 21*(6), 1164-1177.

Meyer, J. G. (2014). In Silico Proteome Cleavage Reveals Iterative Digestion Strategy for High Sequence Coverage. *ISRN Computational Biology*, 2014.

Minard, P., Murrell, K. D., & Stirewalt, M. A. (1977). Proteolytic, antigenic and immunogenic properties of Schistosoma mansoni cercarial secretion material. *The American Journal of Tropical Medicine and Hygiene, 26*(3), 491-499.

Mischak, H., Coon, J. J., Novak, J., Weissinger, E. M., Schanstra, J. P., & Dominiczak, A. F. (2009). Capillary electrophoresis–mass spectrometry as a powerful tool in biomarker discovery and clinical diagnosis: an update of recent developments. *Mass Spectrometry Reviews, 28*(5), 703-724.

Mishur, R. J., & Rea, S. L. (2012). Applications of mass spectrometry to metabolomics and metabonomics: Detection of biomarkers of aging and of age-related diseases. *Mass Spectrometry Reviews, 31*(1), 70-95.

Montenegro, S. M., Miranda, P., Mahanty, S., Abath, F. G., Teixeira, K. M., Coutinho, E. M., & Wynn, T. A. (1999). Cytokine production in acute versus chronic human schistosomiasis mansoni: the cross-regulatory role of interferon-γ and interleukin-10 in the responses of peripheral blood mononuclear cells and splenocytes to parasite antigens. *Journal of Infectious Diseases, 179*(6), 1502-1514.

Morris, G. P., & Threadgold, L. T. (1968). Ultrastructure of the tegument of adult Schistosoma mansoni. *The Journal of Parasitology*, *54*(1), 15-27.

Mueller, L. N., Brusniak, M. Y., Mani, D. R., & Aebersold, R. (2008). An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. *Journal of Proteome Research, 7*(01), 51-61.

Mutapi, F. (2015). Changing Policy and Practice in the Control of Pediatric Schistosomiasis. *Pediatrics, 135*(3), 536-544.

Naus, C. W., Booth, M., Jones, F. M., Kemijumbi, J., Vennervald, B. J., Kariuki, C. H. & Dunne, D. W. (2003). The relationship between age, sex, egg-count and specific antibody responses against Schistosoma mansoni antigens in a Ugandan fishing community. *Tropical Medicine & International Health, 8*(6), 561-568.

Naus, C. W., Kimani, G., Ouma, J. H., Fulford, A. J., Webster, M., van Dam, G. J., ... & Dunne,

D. W. (1999). Development of antibody isotype responses to Schistosoma mansoni in an immunologically naive immigrant population: influence of infection duration, infection intensity, and host age. *Infection and Immunity, 67*(7), 3444-3451.

Ndao, M., Spithill, T. W., Caffrey, R., Li, H., Podust, V. N., Perichon, R. & Ward, B. J. (2010). Identification of novel diagnostic serum biomarkers for Chagas' disease in asymptomatic subjects by mass spectrometric profiling. *Journal of Clinical Microbiology, 48*(4), 1139-1149.

Negrão-Corrêa, D., Fittipaldi, J. F., Lambertucci, J. R., Teixeira, M. M., de Figueiredo Antunes, C. M., & Carneiro, M. (2014). Association of Schistosoma mansoni-Specific IgG and IgE Antibody Production and Clinical Schistosomiasis Status in a Rural Area of Minas Gerais, Brazil. *PloS One, 9*(2), e88042.

Neill, P. J., Smith, J. H., Doughty, B. L., & Kemp, M. (1988). The ultrastructure of the Schistosoma mansoni egg. *The American Journal of Tropical Medicine and Hygiene, 39*(1), 52-65.

Nilsson, T., Mann, M., Aebersold, R., Yates, J. R., Bairoch, A., & Bergeron, J. J. (2010). Mass spectrometry in high-throughput proteomics: ready for the big time. *Nature Methods, 7*(9), 681-685.

Nishioka, Y., Nishimura, N., Suzuki, Y., & Sone, S. (2001). Human monocyte-derived and CD83+ blood dendritic cells enhance NK cell-mediated cytotoxicity. *European Journal of Immunology, 31*(9), 2633-2641.

Nourel, D. M., Nibbeling, R., Rotmans, J. P., Polderman, A. M., Krijger, F. W., & Deelder, A. M. (1994). Quantitative determination of circulating soluble egg antigen in urine and serum of Schistosoma mansoni-infected individuals using a combined two-site enzyme-linked immunosorbent assay. *The American Journal of Tropical Medicine and Hygiene, 50*(5), 585-594.

Ni, J., & Chan, K. (2001). Sequence verification of oligonucleotides by electrospray quadrupole time-of flight mass spectrometry. *Rapid Communications in Mass Spectrometry, 15*(17), 1600-1608.

Oliveira, E. J. D., Kanamura, H. Y., & Lima, D. M. C. (2005). Efficacy of an enzyme-linked

immunosorbent assay as a diagnostic tool for schistosomiasis mansoni in individuals with low worm burden. *Memórias do Instituto Oswaldo Cruz, 100*(4), 421-425.

Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., & Mann, M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell, 127*(3), 635-648.

Olsen, J. V., Schwartz, J. C., Griep-Raming, J., Nielsen, M. L., Damoc, E., Denisov, E. & Wouters, E. R. (2009). A dual pressure linear ion trap Orbitrap instrument with very high sequencing speed. *Molecular & Cellular Proteomics, 8*(12), 2759-2769.

Olveda, D. U., Olveda, R. M., McManus, D. P., Cai, P., Chau, T. N., Lam, A. K. & Ross, A. G. (2014). The chronic enteropathogenic disease schistosomiasis. *International Journal of Infectious Diseases, 28,* 193-203.

Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., & Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Molecular & Cellular Proteomics, 1*(5), 376-386.

Orvisky, E., Drake, S. K., Martin, B. M., Abdel-Hamid, M., Ressom, H. W., Varghese, R. S., & Goldman, R. (2006). Enrichment of low molecular weight fraction of serum for MS analysis of peptides associated with hepatocellular carcinoma. *Proteomics, 6*(9), 2895-2902.

Papadopoulos, M. C., Abel, P. M., Agranoff, D., Stich, A., Tarelli, E., Bell, B. A. & Krishna, S. (2004). A novel and accurate diagnostic test for human African trypanosomiasis. *The Lancet, 363*(9418), 1358-1363.

Patz, J. A., Graczyk, T. K., Geller, N., & Vittor, A. Y. (2000). Effects of environmental change on emerging parasitic diseases. *International Journal for Parasitology, 30*(12), 1395-1405.

Paweletz, C. P., Liotta, L. A., & Petricoin III, E. F. (2001). New technologies for biomarker analysis of prostate cancer progression: Laser capture microdissection and tissue proteomics. Urology, 57(4), 160-163.

Pearce, E. J., & MacDonald, A. S. (2002). The immunobiology of schistosomiasis. *Nature Reviews*

Immunology, 2(7), 499-511.

Pereira, R. V., de Souza Gomes, M., Costa, M. P., Passos, L. K. J., de Castro Borges, W., & Guerra-Sá, R. (2015). MJD and OTU deubiquitinating enzymes in Schistosoma mansoni. *Parasitology research, 114*(8), *2835-2843.*

Pereira, T. A., Syn, W. K., Machado, M. V., Vidigal, P. V., Resende, V., Voieta, I., ... & Diehl, A.

M. (2015). Schistosome-induced cholangiocyte proliferation and osteopontin secretion correlate with fibrosis and portal hypertension in human and murine schistosomiasis mansoni. *Clinical Science, 129*(10), 875-883.

Persidis A. (1998). Proteomics - An ambitious drug development platform attempts to link gene sequence to expressed phenotype under various physiological states. *Nature Biotechnology 16*(4), 393-394.

Petricoin, E. F., Zoon, K. C., Kohn, E. C., Barrett, J. C., & Liotta, L. A. (2002). Clinical proteomics: translating benchside promise into bedside reality. *Nature Reviews Drug Discovery, 1*(9), 683-695.

Pinto-Silva, R. A., Queiroz, L. C. D., Azeredo, L. M., Silva, L. C. D. S., & Lambertucci, J. R. (2010). Ultrasound in schistosomiasis mansoni. *Memórias do Instituto Oswaldo Cruz, 105*(4), 479-484.

Poon, T. C., Chan, K. A., Ng, P. C., Chiu, R. W., Ang, I. L., Tong, Y. K., ... & Lo, Y. D. (2004). Serial analysis of plasma proteomic signatures in pediatric patients with severe acute respiratory syndrome and correlation with viral load. *Clinical Chemistry, 50*(8), 1452-1455.

Popescu, I. D., Codrici, E., Albulescu, L., Mihai, S., Enciu, A. M., Albulescu, R., & Tanase, C. P.

(2014). Potential serum biomarkers for glioblastoma diagnostic assessed by proteomic approaches. *Proteome Science, 12*(1), 47.

Pusch, W., & Kostrzewa, M. (2005). Application of MALDI-TOF mass spectrometry in screening and diagnostic research. *Current pharmaceutical design, 11*(20), 2577-2591.

Rajabi, K., Ashcroft, A. E., & Radford, S. E. (2015). Mass spectrometric methods to analyze the structural organization of macromolecular complexes. *Methods, 89*(1), 13-21.

Ramus, C., Hovasse, A., Marcellin, M., Hesse, A. M., Mouton-Barbosa, E., Bouyssié, D.& de

Peredo, A. G. (2016). Benchmarking quantitative label-free LC–MS data processing workflows using a complex spiked proteomic standard dataset. *Journal of Proteomics, 132,* 51-62.

Ratto-Kim, S., Chuenchitra, T., Pulliam, L., Paris, R., Sukwit, S., Gongwon, S., ... & Valcour, V. G. (2008). Expression of monocyte markers in HIV-1 infected individuals with or without HIV associated dementia and normal controls in Bangkok Thailand. *Journal of Neuroimmunology, 195*(1), 100-107.

Reddy, G., & Dalmasso, E. A. (2003). SELDI ProteinChip® array technology: protein-based predictive medicine and drug discovery applications. *BioMed Research International, 2003*(4), 237-241.

Reyman, T. A., Zimmerman, M. R., & Lewin, P. K. (1977). Autopsy of an Egyptian mummy. 5. Histopathologic investigation. *Canadian Medical Association Journal, 117*(5), 470.

Richter, J., Domingues, A. L. C., Barata, C. H., Prata, A. R., & Lambertucci, J. R. (2001). Report of the second satellite symposium on ultrasound in schistosomiasis. *Memórias do Instituto Oswaldo Cruz, 96*, 151-156.

Rioux, M. C., Carmona, C., Acosta, D., Ward, B., Ndao, M., Gibbs, B. F. & Spithill, T. W. (2008). Discovery and validation of serum biomarkers expressed over the first twelve weeks of Fasciola hepatica infection in sheep. *International Journal for Parasitology, 38*(1), 123-136.

Roberts, M., Butterworth, A. E., Kimani, G., Kamau, T., Fulford, A. J., Dunne, D. W. & Sturrock, R. F. (1993). Immunity after treatment of human schistosomiasis: association between cellular responses and resistance to reinfection. *Infection and Immunity, 61*(12), 4984-4993.

Rohmer, M., Baeumlisberger, D., Stahl, B., Bahr, U., & Karas, M. (2011). Fragmentation of neutral oligosaccharides using the MALDI LTQ Orbitrap.*International Journal of Mass Spectrometry*, *305*(2), 199-208.

Rosenberg, H. F. (1998). The eosinophil ribonucleases. *Cellular and Molecular Life Sciences CMLS, 54*(8), 795-803.

Ross, A.G.P., Bartley, P.B., Sleigh, A.C., et al. (2002). Schistosomiasis. *The New England Journal of Medicine*, *346*(16):1212–1220.

Rossi, E., Adams, L. A., Bulsara, M., & Jeffrey, G. P. (2007). Assessing liver fibrosis with serum

marker models. *Clinical Biochemist Reviews, 28*(1), 3.

Rumbley, C. A., Sugaya, H., Zekavat, S. A., El Refaei, M., Perrin, P. J., & Phillips, S. M. (1999). Activated eosinophils are the major source of Th2-associated cytokines in the schistosome granuloma. *The Journal of Immunology, 162*(2), 1003-1009.

Ryzhov, V., & Fenselau, C. (2001). Characterization of the protein subset desorbed by MALDI from whole bacterial cells. *Analytical Chemistry, 73*(4), 746-750.

Salter, J. P., Choe, Y., Albrecht, H., Franklin, C., Lim, K. C., Craik, C. S., & McKerrow, J. H. (2002). Cercarial elastase is encoded by a functionally conserved gene family across multiple species of schistosomes. *Journal of Biological Chemistry, 277*(27), 24618-24624.

Salzet, M., Capron, A., & Stefano, G. B. (2000). Molecular Crosstalk in Host–Parasite Relationships: Schistosome–and Leech–Host Interactions. Parasitology Today, 16(12), 536-540.

Sandoval, N., Siles-Lucas, M., Aban, J. L., Pérez-Arellano, J. L., Gárate, T., & Muro, A. (2006). Schistosoma mansoni: A diagnostic approach to detect acute schistosomiasis infection in a murine model by PCR. *Experimental Parasitology, 114*(2), 84-88.

Sampath, R., Mulholland, N., Blyn, L. B., Massire, C., Whitehouse, C. A., Waybright, N. & Ecker, D. J. (2012). Comprehensive biothreat cluster identification by PCR/electrospray-ionization mass spectrometry. *PLoS One, 7*(6), e36528.

Savioli, L., Fenwick, A., Rollinson, D., Albonico, M., & Ame, S. M. (2015). An achievable goal: control and elimination of schistosomiasis. *The Lancet, 386*(9995), 739.

Schaub, S., Rush, D., Wilkins, J., Gibson, I. W., Weiler, T., Sangster, K., & Nickerson, P. (2004). Proteomic-based detection of urine proteins associated with acute renal allograft rejection. *Journal of the American Society of Nephrology, 15*(1), 219-227.

Scheld, M. W., W Michael Scheld, M. D., Whitley, R. J., & Marra, C. M. (2014). Infections of the

central nervous system. Lippincott Williams & Wilkins.

Scherl, A. (2015). Clinical protein mass spectrometry. *Methods, 81, 3-14.*

Schistosomiasis. Centers for Disease Control, 2009. Web. <http://www.dpd.cdc.gov/dpdx/html/schistosomiasis.htm>.

Schulze, W. X., & Mann, M. (2004). A novel proteomic screen for peptide-protein interactions.

Journal of Biological Chemistry, 279(11), 10756-10764.

Schwegler, E. E., Cazares, L., Steel, L. F., Adam, B. L., Johnson, D. A., Semmes, O. J., & Drake, R. R. (2005). SELDI-TOF MS profiling of serum for detection of the progression of chronic hepatitis C to hepatocellular carcinoma. *Hepatology, 41*(3), 634-642.

Schwingshackl, A., Duszyk, M., Brown, N., & Moqbel, R. (1999). Human eosinophils release matrix metalloproteinase-9 on stimulation with TNF-α. *Journal of Allergy and Clinical Immunology, 104*(5), 983-990.

Sher, A. L. A. N., Coffman, R. L., Hieny, S., & Cheever, A. W. (1990). Ablation of eosinophil and

IgE responses with anti-IL-5 or anti-IL-4 antibodies fails to affect immunity against Schistosoma mansoni in the mouse. *The Journal of Immunology, 145*(11), 3911-3916.

Shevchenko, G., Konzer, A., Musunuri, S., & Bergquist, J. (2015). Neuroproteomics tools in clinical practice. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1854*(7), 705-717.

Shiha, G., Sarin, S. K., Ibrahim, A. E., Omata, M., Kumar, A., Lesmana, L. A. & Elwakil, R. (2009). Liver fibrosis: consensus recommendations of the Asian Pacific Association for the Study of the Liver (APASL). *Hepatology International, 3*(2), 323-333.

Shinkai, K., Mohrs, M., & Locksley, R. M. (2002). Helper T cells regulate type-2 innate immunity

in vivo. *Nature, 420*(6917), 825-829.

Seibert, V., Wiesner, A., Buschmann, T., & Meuer, J. (2004). Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and ProteinChip® technology in proteomics research. *Pathology-Research and Practice, 200*(2), 83-94.

Selbach, M., & Mann, M. (2006). Protein interaction screening by quantitative immunoprecipitation combined with knockdown (QUICK). *Nature Methods, 3*(12), 981-983.

Simner, P. J., Uhl, J. R., Hall, L., Weber, M. M., Walchak, R. C., Buckwalter, S., & Wengenack, N. L. (2013). Broad-range direct detection and identification of fungi by use of the PLEX-ID PCR-electrospray ionization mass spectrometry (ESI-MS) system. *Journal of Clinical Microbiology, 51*(6), 1699-1706.

Sinkala, E., Kapulu, M. C., Besa, E., Zyambo, K., Chisoso, N. A. J., Foster, G. R., & Kelly, P.

(2015). Hepatosplenic schistosomiasis is characterised by high blood markers of translocation, inflammation and fibrosis. *Liver International*, *36*(1), 145-150.

Siuzdak, G., Press, M. C. C., & Schalley, C. A. (2004). The expanding role of mass spectrometry in biotechnology. *Journal of American Society for Mass Spectrometry, 15,* 625.

Skelly, P. J. (2013). The use of imaging to detect schistosomes and diagnose schistosomiasis. *Parasite Immunology, 35*(9-10), 295-301.

Skelly, P. J., Kim, J. W., Cunningham, J., & Shoemaker, C. B. (1994). Cloning, characterization, and functional expression of cDNAs encoding glucose transporter proteins from the human parasite Schistosoma mansoni. *Journal of Biological Chemistry, 269*(6), 4247-4253.

Sotillo, J., Pearson, M., Becker, L., Mulvenna, J., & Loukas, A. (2015). A quantitative proteomic analysis of the tegumental proteins from Schistosoma mansoni schistosomula reveals novel

potential therapeutic targets. *International Journal for Parasitology, 45*(8), 505-516.

Stirewalt, M. A. (1963). Cercaria vs. schistosomule (Schistosoma mansoni): absence of the pericercarial envelope in vivo and the early physiological and histological metamorphosis of the parasite. *Experimental Parasitology, 13*(3), 395-406.

Stirewalt, M. A. (1974). Schistosoma mansoni: cercaria to schistosomule. *Adv Parasitol, 12,* 115-

182.

Stothard, J. R., Kabatereine, N. B., Tukahebwa, E. M., Kazibwe, F., Rollinson, D., Mathieson, W., & Fenwick, A. (2006). Use of circulating cathodic antigen (CCA) dipsticks for detection of intestinal and urinary schistosomiasis. *Acta Tropica, 97*(2), 219-228.

Stothard, J. R., Sousa-Figueiredo, J. C., Standley, C., Van Dam, G. J., Knopp, S., Utzinger, J., ... & Rollinson, D. (2009). An evaluation of urine-CCA strip test and fingerprick blood SEA-ELISA for detection of urinary schistosomiasis in schoolchildren in Zanzibar. *Acta Tropica*, *111*(1), 64-70.

Swartz, J. M., Byström, J., Dyer, K. D., Nitto, T., Wynn, T. A., & Rosenberg, H. F. (2004). Plasminogen activator inhibitor-2 (PAI-2) in eosinophilic leukocytes. *Journal of Leukocyte biology, 76*(4), 812-819.

Swartz, J. M., Dyer, K. D., Cheever, A. W., Ramalingam, T., Pesnicak, L., Domachowske, J. B.,

... & Rosenberg, H. F. (2006). Schistosoma mansoni infection in eosinophil lineage–ablated mice. *Blood, 108*(7), 2420-2427.

Theodorescu, D., & Mischak, H. (2007). Mass spectrometry based proteomics in urine biomarker discovery. *World Journal of Urology, 25*(5), 435-443.

Thevis, M., Thomas, A., & Schänzer, W. (2008). Mass spectrometric determination of insulins and their degradation products in sports drug testing. *Mass Spectrom Rev 27*:35–50.

Thompson, D. P., & Geary, T. G. (2003). Helminth surfaces: structural, molecular and functional properties. *Molecular Medical Parasitology,* 297-338.

Tran, M. H., Pearson, M. S., Bethony, J. M., Smyth, D. J., Jones, M. K., Duke, M., & Loukas, A. (2006). Tetraspanins on the surface of Schistosoma mansoni are protective antigens against schistosomiasis. *Nature Medicine, 12*(7), 835-840.

Utzinger, J., N'Goran, E. K., Caffrey, C. R., & Keiser, J. (2011). From innovation to application: social–ecological context, diagnostics, drugs and integrated control of schistosomiasis. *Acta Tropica, 120*, S121-S137.

Vaidyanathan, S., Winder, C. L., Wade, S. C., Kell, D. B., & Goodacre, R. (2002). Sample preparation in matrix-assisted laser desorption/ionization mass spectrometry of whole bacterial cells and the detection of high mass (> 20 kDa) proteins. *Rapid Communications in Mass Spectrometry, 16*(13), 1276-1286.

Valaskovic, G. A., Kelleher, N. L., Little, D. P., Aaserud, D. J., & Mclafferty, F. W. (1995). Attomole-sensitivity electrospray source for large-molecule mass spectrometry. *Analytical Chemistry, 67*(20), 3802-3805.

Van Dam, G. J., Wichers, J. H., Ferreira, T. F., Ghati, D., Van Amerongen, A., & Deelder, A. M. (2004). Diagnosis of schistosomiasis by reagent strip test for detection of circulating cathodic antigen. *Journal of Clinical Microbiology, 42*(12), 5458-5461.

Van Lieshout, L., Polderman, A. M., & Deelder, A. M. (2000). Immunodiagnosis of schistosomiasis by determination of the circulating antigens CAA and CCA, in particular in individuals with recent or light infections. *Acta Tropica, 77*(1), 69-80.

Verweij, J. J., Blangé, R. A., Templeton, K., Schinkel, J., Brienen, E. A., van Rooyen, M. A., ... & Polderman, A. M. (2004). Simultaneous detection of Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum in fecal samples by using multiplex real-time PCR. *Journal of Clinical Microbiology, 42*(3), 1220-1223.

Verweij, J. J., Brienen, E. A., Ziem, J., Yelifari, L., Polderman, A. M., & Van Lieshout, L. (2007). Simultaneous detection and quantification of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in fecal samples using multiplex real-time PCR. *The American Journal of Tropical Medicine and Hygiene, 77*(4), 685-690.

Vezozzo, D. C. P., Farias, A. Q., Cerri, G. G., Da Silva, L. C., & Carrilho, F. J. (2006). Assessment of portal hemodynamics by Doppler ultrasound and of liver morphology in the hepatosplenic and hepatointestinal forms of schistosomiasis mansoni. *Digestive Diseases and Sciences, 51*(8), 1413-1419.

Vianna, M. R., Gayotto, L. C. C., Telma, R., Santos, M., Alves, V. A., Fukushima, J., & Brito, T. (1989). *Intrahepatic bile duct changes in human hepatosplenic schistosomiasis mansoni. Liver, 9*(2), 100-109.

Villanueva, J., Shaffer, D. R., Philip, J., Chaparro, C. A., Erdjument-Bromage, H., Olshen, A. B., ... & Tempst, P. (2006). Differential exoprotease activities confer tumor-specific serum peptidome patterns. *The Journal of Clinical Investigation, 116*(1), 271-284.

Vlahou, A., Schellhammer, P. F., Mendrinos, S., Patel, K., Kondylis, F. I., Gong, L., & Wright Jr, G. L. (2001). Development of a novel proteomic approach for the detection of transitional cell carcinoma of the bladder in urine. *The American Journal of Pathology, 158*(4), 1491-1502.

Waanders, L. F., Hanke, S., & Mann, M. (2007). Top-down quantitation and characterization of SILAC-labeled proteins. *Journal of the American Society for Mass Spectrometry, 18*(11), 2058-2064.

Wan, K. X., Gross, M. L., & Shibue, T. (2000). Gas-phase stability of double-stranded oligodeoxynucleotides and their noncovalent complexes with DNA-binding drugs as revealed by collisional activation in an ion trap. *Journal of the American Society for Mass Spectrometry, 11*(5), 450-457.

Wang, L., Utzinger, J., & Zhou, X. N. (2008). Schistosomiasis control: experiences and lessons from China. *The Lancet, 372*(9652), 1793-1795.

Warren, K. S. (1978). The pathology, pathobiology and pathogenesis of schistosomiasis. *Nature, 273*(5664), 609-612.

Washburn, M. P., Wolters, D., & Yates, J. R. (2001). Large-scale analysis of the yeast proteome

by multidimensional protein identification technology. *Nature Biotechnology, 19*(3), 242-247.

Weerakoon, K. G., Gobert, G. N., Cai, P., & McManus, D. P. (2015). Advances in the Diagnosis of Human Schistosomiasis. *Clinical Microbiology Reviews, 28*(4), 939-967.

Wei, O. (1973). Internal organs of a 2100-year-old female corpse. *The Lancet, 302*(7839), 1198.

Wei, J., Buriak, J. M., & Siuzdak, G. (1999). Desorption–ionization mass spectrometry on porous silicon. *Nature, 399*(6733), 243-246.

World Health Organization. (2002). Prevention and control of schistosomiasis and soil-transmitted

helminthiasis.

World Health Organization. (2012). Accelerating work to overcome the global impact of neglected

tropical diseases: a roadmap for implementation: executive summary.

Williams, T. L., Andrzejewski, D., Lay Jr, J. O., & Musser, S. M. (2003). Experimental factors affecting the quality and reproducibility of MALDI-TOF mass spectra obtained from whole bacteria cells. *Journal of the American Society for Mass Spectrometry, 14*(4), 342-351.

Wilson, R. A., Ashton, P. D., Braschi, S., Dillon, G. P., Berriman, M., & Ivens, A. (2007). 'Oming in on schistosomes: prospects and limitations for post-genomics. *Trends in Parasitology, 23*(1), 14-20.

Wilson, R. A., & Barnes, P. E. (1974). The tegument of Schistosoma mansoni: observations on the formation, structure and composition of cytoplasmic inclusions in relation to tegument function. *Parasitology, 68*(02), 239-258.

Wilson, M. S., & Maizels, R. M. (2004). Regulation of allergy and autoimmunity in helminth infection. *Clinical Reviews in Allergy & Immunology, 26*(1), 35-50.

Wong, C. C., Cociorva, D., Venable, J. D., Xu, T., & Yates III, J. R. (2009). Comparison of different signal thresholds on data dependent sampling in orbitrap and LTQ mass spectrometry for the identification of peptides and proteins in complex mixtures. *Journal of the American Society for Mass Spectrometry, 20*(8), 1405-1414.

Wu, L., Wu, M., Tian, D., Chen, S., Liu, B., Chen, Q. & Guo, Z. (2012). Clinical and imaging

characteristics of cerebral schistosomiasis. *Cell Biochemistry and Biophysics, 62*(2), 289-295.

Xiao, S. H., & Catto, B. A. (1989). In vitro and in vivo studies of the effect of artemether on Schistosoma mansoni. *Antimicrobial Agents and Chemotherapy, 33*(9), 1557-1562.

Xu, J., Liu, A. P., Guo, J. J., Wang, B., Qiu, S. J., Sun, H., ... & Wu, Z. D. (2013). The sources and metabolic dynamics of Schistosoma japonicum DNA in serum of the host. *Parasitology Research*, *112*(1), 129-133.

Yates, J. R., Ruse, C. I., & Nakorchevsky, A. (2009). Proteomics by mass spectrometry: approaches, advances, and applications. *Annual Review of Biomedical Engineering, 11*, 49-79.

Yoshino, T. P., & Bayne, C. J. (1983). Mimicry of snail host antigens by miracidia and primary sporocysts of Schistosoma mansoni. *Parasite Immunology, 5*(3), 317-328.

Yu, Y., Hagihara, M., Ando, K., Gansuvd, B., Matsuzawa, H., Tsuchiya, T., & Kato, S. (2001). Enhancement of human cord blood CD34+ cell-derived NK cell cytotoxicity by dendritic cells. *The Journal of Immunology, 166*(3), 1590-1600.

Zhao, Y., Chang, C., Qin, P., Cao, Q., Tian, F., Jiang, J., ... & Ying, W. (2016). Mining the human

plasma proteome with three-dimensional strategies by high-resolution Quadrupole Orbitrap Mass Spectrometry. *Analytica Chimica Acta*, 904, 65-75.

Zhang, Z., & Shah, B. (2007). Characterization of variable regions of monoclonal antibodies by top-down mass spectrometry. *Analytical Chemistry, 79*(15), 5723-5729.

Zhou, Y. B., Zheng, H. M., & Jiang, Q. W. (2011). A diagnostic challenge for schistosomiasis japonica in China: consequences on praziquantel-based morbidity control. *Parasit Vectors*, *4*, 194.

Zybailov, B., Coleman, M. K., Florens, L., & Washburn, M. P. (2005). Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labeling. *Analytical Chemistry, 77*(19), 6218-6224.

CHAPTER II

Identification of Candidate Serum Biomarkers for *Schistosoma mansoni* Infected Mice Using Multiple Proteomic Platforms

Manal I. Kardoush[1, 2, 3], Brian J. Ward[2, 4], Momar Ndao[2, 4*]

Affiliations:

[1]Institute of Parasitology, McGill University, Montreal, Quebec, Canada

[2] National Reference Centre for Parasitology, Research Institute of the McGill University Health Centre, Montreal General Hospital, Montreal, Quebec, Canada

[3]Department of Parasitology, Faculty of medicine, Benha University, Benha, Qalubia, Egypt

[4]JD MacLean Tropical Diseases Centre, Research Institute of the McGill University Health Centre, Montreal General Hospital, Montreal, Quebec, Canada

* Corresponding Authour

Momar Ndao

Research Institute of the McGill University Health Center

Glen site – EM3-3248

1001 Décarie Blvd,

Montréal, QC,

H4A 3J1

Telephone: 514-934-1934 x44537

FAX: 514-934-8347

Email: momar.ndao@mcgill.ca

Abstract

Background: Schistosomiasis is an important helminth infection of humans. There are few reliable diagnostic biomarkers for early infection, for recurrent infection or to document successful treatment. In this study, we compared serum protein profiles in uninfected and infected mice to identify disease stage-specific biomarkers. **Methods:** Serum collected from CD1 mice infected with 50-200 *Schistosoma mansoni* cercariae were analyzed before infection and at 3, 6 and 12 weeks post-infection using three mass spectrometric (MS) platforms. **Results:** Using SELDI-TOF MS, 66 discriminating m/z peaks were detected between *S. mansoni* infected mice and healthy controls. Used in various combinations, these peaks could 1) reliably diagnose early-stage disease, 2) distinguish between acute and chronic infection and 3) diagnose *S. mansoni* infection regardless the parasite burden. The most important contributors to these diagnostic algorithms were peaks at 3.7, 13 and 46 kDa. Employing sample fractionation and differential gel electrophoresis, we analyzed gel slices either by MALDI-TOF MS or Velos Orbitrap MS. The former yielded eight differentially-expressed host proteins in the serum at different disease stages including transferrin and alpha 1- antitrypsin. The latter suggested the presence of a surprising number of parasite-origin proteins in the serum during both the acute (n=200) and chronic (n=105) stages. The Orbitrap platform also identified many differentially-expressed host-origin serum proteins during the acute and chronic stages (296 and 220 respectively). The presence of one of the schistosome proteins, glutathione S-transferase (GST: 25 KDa), was confirmed by Western blot. This study provides proof-of-principle for an approach that can yield a large number of novel candidate biomarkers for *Schistosoma* infection.

Keywords: *Schistosoma mansoni*, diagnostics, proteomics, biomarkers, mouse model.

Word Counts: Abstract: 259

Text: 4,813

6 Figures/ 3 Tables

Introduction

Schistosomiasis is a public health problem of global importance (Hotez & Fenwick, 2009). For both surveillance and the optimal treatment of patients, rapid and sensitive diagnostic tests are needed that can detect infection soon after exposure and when parasite burden is low. Although the current gold-standard test for *Schistosoma mansoni* is microscopic detection of the eggs in stool, eggs first appear only 6-8 weeks after infection. This method also has poor sensitivity when few parasites are present and during the chronic stage of infection when the passage of eggs is typically low (Jordan & Webbe, 1969). Other tools used to diagnose and monitor schistosomiasis include the detection of circulating antigens or antibodies and ultrasound to assess liver fibrosis and hepatosplenomegaly (Deelder et al., 1980; Doenhoff et al., 2004; Greter et al., 2015; Richter et al., 1998). Polymerase chain reaction (PCR) has recently been used to detect *S. mansoni* DNA in human fecal samples (Espírito-Santo et al., 2015; Pontes & Rabello, 2002). All of these tests have important limitations related to their complexity, expense, sensitivity and/or cross-reactivity with other helminth infections. Most cannot discriminate between active and past infections (Bergquist et al., 2009; Gryseels et al., 2006). Therefore, there is a need for new schistosomiasis diagnostic options.

Mass spectrometry (MS) has the potential to modernize *S. mansoni* diagnostics through the discovery of specific biomarkers or proteomic profiles associated with infection or disease stage. In addition to possible diagnostic advances, the application of MS techniques to serum samples from the well-described schistosome-infected mouse model also has the potential to provide novel insights into parasite biology. Although the choice of proteomic platform and the

optimal timing of sampling were unknown at the launch of these studies, we hoped to identify candidate biomarkers at different time-points after infection, representing the different pathological stages of the disease: ie: EARLY prior to egg production (~3 weeks post-infection), ACUTE: ~6 weeks post-infection when eggs are being starting to be deposited in the liver, and CHRONIC: ~12 weeks post-infection when there is a well-defined granulomatous reaction in the liver.

Because several different MS platforms are available, each with its particular strengths and weaknesses, we opted to explore three complimentary approaches. We used high throughput surface-enhance, laser-desorption and ionization, time-of-flight mass spectroscopy (SELDI-TOF MS) to compare uninfected and infected sera as a 'proof-of-principal' exercise. We subsequently used sample fractionation and differential gel electrophoresis prior to analysis on two more precise MS platforms; specifically matrix-assisted, laser-desorption and ionization (MALDI-TOF MS) and Velos Orbitrap MS. By using multiple proteomic platforms in parallel, we demonstrated that serum protein profiles differ extensively between infected and uninfected mice, offering a rich source of potential biomarkers.

Materials and Methods

Mouse infection and serum collection

Twenty-six female CD1, six-week old mice were purchased from The Charles River (St. Constant, Québec). All animal experiments were approved by the Facility Animal Care Committee of McGill University and followed the guidelines of the Canadian Council on Animal Care. Mice (5/group) were infected intraperitoneally (IP) with 50, 100, 150 or 200 *S. mansoni* cercariae (*S. mansoni*-infected *Biomphalaria glabrata* snails were obtained from the Biomedical Research Institute; Bethesda, MD). Control animals (n=6) were IP injected with PBS. Blood

samples were collected by saphenous bleeding before infection, at three weeks and six weeks post-infection and by cardiac puncture at 12 weeks post-infection. All sera were kept at − 80 °C until analyzed.

Fractionation method:

We used sample fractionation prior to SELDI and MALDI analysis. Serum samples at different time points were fractionated as previously described (Rioux et al., 2008; Santamaria et al., 2014). Briefly, ProteinChip serum fractionation kit (Bio-Rad) was used to fractionate the samples into six pH fractions prior to mass spectrometric analysis. 96-well Q-Ceramic HyperD F resin filtration plates were used for the fractionation. To decrease the complexity, 20 µL of sample were first incubated with 30 µL of U9 buffer (9 M urea 2% CHAPS, and 50 mM Tris-HCl, pH 9) for 20 minutes. Then, samples were diluted with 50 µL of U1 buffer [1 M urea, 2% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM Tris-HCl, pH 9] and added to pre-equilibrated filtration plates incubated on a MicroMix machine (MicroMix 5; Diagnostic Products Company, Los Angeles, CA) for 30 minutes. Five fractions were collected by serial elution in buffers of decreasing pH (pH9, 7, 5, 4, 3). The buffers used are wash buffer 1 (50 mM Tris-HCl, 0.1% OGP, pH 9), wash buffer 2 (50 mM HEPES, 0.1% OGP, pH 7), wash buffer 3 (100 mM Na acetate, 0.1% OGP, pH 5), wash buffer 4 (100 mM Na acetate, 0.1% OGP, pH 4) and wash buffer 5 (50 mM Na citrate, 0.1% OGP, pH 3). After the collection of the first five fractions, a final elution by vacuum in an organic buffer was collected. The buffer used in the final collection was wash buffer 6 (33.3% isopropanol, 16.7% acetonitrile, 0.1% trifluoroacetic acid). All fractions were stored at -80 ºC until used.

SELDI-TOF MS

SELDI-TOF MS was introduced by Hutchens T.W. and Yip T.T in 1993 and has many potential advantages including high throughput, small volume requirement, a broad dynamic range and the capacity to analyze complex samples such as serum by pre-fractionation on chromatographically-distinct 'chips'. The fractionated serum samples were bound to ProteinChip™ arrays (Bio-Rad) and analyzed essentially as previously described (Rioux et al., 2008; Santamaria et al., 2014). Cation Exchange (CM10) and Immobilized Metal Affinity Capture (IMAC30) Protein-Chip™ arrays were used to profile the fractionated samples as previously described (Santamaria et al., 2014). Each chip type was read under high and low laser intensity (3750 and 2500 nanojoules respectively). Biomarker Patterns Software™ algorithms (Bio-Rad) were then used to analyze the peak intensities and to generate classification 'trees' by CART analysis (Bio-Rad Laboratories) (Steinberg & Colla, 1995). This experiment suggested that the greatest number of potential biomarkers were present in fractions 1 and 6 (F1, F6).

ProteinChip Data Manager Software:

ProteinChip Data Manager Software (version 3.5; Bio-Rad) was used first to determine protein clustering. The latter defines a group of peaks of similar mass that are treated as the same protein or peptide across multiple spectra. The peaks generated within an MS spectrum indicate signal intensity on the y-axis and mass-to-ion ratio (m/z) on the x-axis. The same peptide or protein peaks with different levels from each serum sample were collected and grouped into clusters. Two-step spectral analysis was performed. In the first pass analysis, automated detection identified peaks using a signal-to-noise ratio (S/N) of 5, and cluster peaks with a p-values ≤ 0.05 were inspected and relabeled manually. In the second pass analysis, peak detection was performed on the user-defined peaks only, and peaks with S/N ratio ≥ 2 were retained. The Biomarker Wizard application of the ProteinChip™ software was then used to compare the data

between control and infected groups using the Mann–Whitney test. Potential biomarkers were defined as peaks with p-values of ≤ 0.05 and Receiver Operation Characteristic (ROC) values of < 0.3 and > 0.7.

Classification and regression tree [CART] Analysis

Biomarker Pattern Software™ (BPS) (Bio-Rad Laboratories) was used to analyze the SELDI peak intensities and generate classification trees (i.e., potential diagnostic algorithms). Using multiple differentially-expressed protein peaks in the sera of infected and control groups, the BPS software creates a series of decision-points at cut-off intensities assigned to achieve the best separation. Sera with values <X go to the left node while those with values ≥X go to the right node. The software repeats this splitting process for each daughter node using other candidate biomarker peaks until terminal nodes are produced (Steinberg & Colla, 1995). CART analysis of SELDI data has been used, among other applications, to identify candidate diagnostic biomarkers in Chagas disease (Ndao et al., 2009), to differentiate hepatocellular carcinoma patients from cirrhotic subjects (Chen et al., 2010), and for diagnosis of lung adenocarcinoma (Lin et al., 2012).

SDS-PAGE and in-gel digestion

The fractionated pooled serum samples (1 μL/lane) from control (12 weeks) as well as acutely (6 weeks) and chronically infected mice (12 weeks) were loaded onto 4-12% Bis-TrisNuPAGE gels (Invitrogen Life Technologies, Carlsbad, CA), and run for 45 min at 200 V. After which the gel was with silver stain (silver staining kit; Bio-Rad). Gel images were captured by scanning and then comparing the intensities of stained spots to compare the amount of specific proteins visually and certain spots were selected for analysis. Gel bands of differentially-expressed proteins (n=45) were excised.

Protein identification by mass spectrometry

Prior to MS analysis, all the gel pieces were dried under the vacuum and then rehydrated in Trypsin Digest Solution (proteomics grade, Roche) at 37°C overnight. Peptides were eluted in a total volume of 5 µl of acetonitrile and 0.1 % trifluoro acetic acid (TFA) solution (50:50 v/v), after desalting with Millipore's C18 Zip Tips, the samples were resuspended in 10 µL 0.1% TFA and subjected to MALDI MS analysis.

MALDI-TOF MS

MALDI-TOF MS is a powerful technique that has been used for protein identification from different biological samples (Lovric, 2011). The resulting digests were analyzed by 4800 Plus MALDI-TOF/TOF analyzer ABSciex (Applied Biosystems, Foster City, CA), as previously described (Santamaria et al., 2014). Briefly, the samples were eluted in alpha-cyano-4-hydroxycinnamic acid matrix (Sigma-Aldrich, St Louis, MO). One µL aliquots was spotted directly onto a 384-well Opti-TOF MALDI stainless steel plates (AB Sciex, Framingham, MA) and allowed to air-dry at room temperature. Ions were generated by pulsing the mixture with a nitrogen laser (Laser energy at 4000 nanojoules). The collected spectra were analyzed using ProteinPilot™ software (Applied Biosystems) by searching the mouse and *S. mansoni* Swiss-Prot protein databases. The enzyme of cleavage was trypsin, fixed modification was carbamidomethyl, and variable modification was oxidation. The confidence threshold for protein identification was set to 95%.

LTQ-Orbitrap Velos

LTQ-Orbitrap mass analyzers can analyze thousands of proteins with high resolution, and high mass accuracy. This approach is useful for identification of unknown proteins even at low concentrations in complex body fluids (Tamura et al., 2105). We used LTQ-Orbitrap Velos

analysis primarily to pursue schistosomal antigens in the infected sera (Clinical Proteomics Platform, McGill University). One µL of pooled serum from control (12 weeks), acutely (6 weeks) and chronically infected mice (12 weeks) were loaded onto precast 10% mini-protean TGX precast gels (BioRad, Mississauga, ON) and run for 45 min at 200 V. The protein gel spots were visualized by staining with Coomassie blue gels (BioRad) for at least 1 h and destained with 10% acetic acid for 30 min. Twenty horizontal bands were excised from each lane, placed in 1.5 mL tubes and exposed to 40 µL of 100 mM NH_4HCO_3 for 5 minutes then centrifuged for 10 min at 14,000 × g, and 4 °C. After aspiration of the liquid, the gel pieces were vortexed with 40 µL of 100 mM NH_4HCO_3/50% acetonitrile - for 10 minutes to shrink the gel. The gel pieces were dried in a microplate at 60°C in a vacuum concentrator for 30 minutes to remove excess acetonitrile and then placed in 40 uL of 10 mM dithiothreitol (DTT)/100 mM $NH4HCO_3$ (Sigma-Aldrich, St Louis, MO) at 56°C in a closed water bath for 60 min. Gel pieces were then alkylated in 40 uL of 55 mM iodoacetamide + 100 mM NH_4HCO_3 for 45 min in 1.5 mL tubes in the dark at room temperature. After centrifugation at 14,000 x g for 15 min, the fluid was aspirated and gel pieces were washed in the same 1.5 mL tube in 100 uL of 100 mM NH_4HCO_3. Tubes were then centrifuged at 14,000 x g for 10 min, followed by removal of the fluid and washing of the gel in 40 uL of 100 mM NH_4HCO_3 for 5 min at room temperature after which 40 uL of acetonitrile (ACN) was added to make 1:1 solution and incubation was continued for 15 min at room temperature. This last wash-step with 100 uL of 25mM NH_4HCO_3 in 50% ACN was repeated x1 then the gel pieces were dried (60°C as above) and digested with 10-20 uL (enough to cover pieces) of trypsin (Roche, Mannheim, Germany) overnight at 37°C (12.5 ng/µL). After digestion, peptides were extracted with 100 µL 1% formic acid and vortexing at 37°C for 15 min. Tubes were then centrifuged at 14,000 x g for 15 min, and the supernatants were transferred to

new 1.5 mL tubes (first extraction). A second extraction was performed with 100 µL 5% formic acid/50% acetonitrile, (Sigma-Aldrich, St Louis, MO) and, after centrifugation (14,000 x g for 15 minutes), the two extraction supernatants were pooled. The pooled supernatants were then dried in a vacuum centrifuge (Hermlelabortechnik, Germany) for ~1-2 hours at 50°C. Dried samples were re-suspended in 40 µL 0.05% formic acid and stored at −20°C until used for MS analysis.

Western Blots

Neat sera samples (1 µL/lane) from control (12 weeks) and acutely (6 weeks) or chronically infected mice (12 weeks) were loaded onto 4-12% Bis-TrisNuPAGE gels (Invitrogen Life Technologies, Carlsbad, CA), and run for 45 min at 200 V. Isolated proteins were transblotted onto nitrocellulose membrane (Invitrogen, KiryatShmona, Israel) at 100 V for seven min and then stained with Ponceau S (Sigma-Aldrich) to verify transfer and as an initial loading control (Aldridge et al., 2008; Welinder et al., 2011). Membranes were blocked (5% skim milk in 0.05% Tween 20 in PBS: PBST buffer) for 1 hour at room temperature (RT), followed by three washes with PBST for 5 min each. Membranes were incubated overnight at 4 °C with the following antibodies: (1) mouse anti-α1-antitrypsin monoclonal antibody (1:500 - Pierce Biotechnology, Rockford, IL) (2) rabbit anti-transferrin polyclonal antibody (1:500 - Santa Cruz Biotechnology) (3) rabbit polyclonal antibody against *S. japonicum* GST (1:500 - US biological) (4) mouse anti-actin monoclonal antibody (1:000 - Santa Cruz Biotechnology). The membranes were washed three times with PBST for 5 min each and incubated with horseradish peroxidase-labeled respective anti-mouse, anti-rabbit, and anti-mouse secondary antibodies (Amersham Biosciences Co., Piscataway, NJ) diluted in 5% non-fat dry milk in PBST (1:10000) for 1 h at room temperature. The membranes were washed one time in PBST for 15 minutes followed by 2 washes for 5 minutes each then incubated in Super Signal West Pico detection solution (Pierce,

Rockford, IL) and exposed to X-ray film. The ratio between the targeted proteins and the actin control bands were used to standardize across samples (Pfaffl, 2001; Liu & Xu 2006). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal loading control. Membranes were stripped with 100 ml of stripping solution: 2% sodium dodecyl sulfate (SDS), 62.5 mM tris HCL pH 6.7, 100 mM mercaptoethanol for 30 minutes at 55°C followed by re-probing with a mouse anti-GAPDH monoclonal (1:5000, Abcam). Blots were incubated with HRP-conjugated anti-mouse IgG at 1:10,000 at RT for 1 h (Amersham Biosciences Co.) and exposed to X-ray film. Image J software (National Institutes of Health, Bethesda, MD) was used to analyze densities of selected bands.

Results

Screening of serum biomarkers in early, acute, and chronic stages of *S. mansoni* infection

In the samples analyzed by SELDI-TOF-MS, a total of 66 candidate biomarkers corresponding to peptides and proteins between 2.9 kDa and 82.3 kDa were identified at the three time points (versus control sera). All were statistically significant (p-value ≤ 0.05) with ROC (Receiver Operation Characteristic) curve values either $0.3<$ or >0.7. Among the 66 differentiating peaks, a 3720 Da protein ($p< 0.006$) was one of the most interesting and potentially useful. The SELDI data indicated that 3720 Da protein was highly over-expressed in early infection (versus control: Fig 1A) making it a potential candidate for identifying the early stage of schistosomiasis. A second biomarker at 13,407 Da ($p< 0.01$) reliably differentiated acute from chronic infection (6 versus 12 weeks: Fig 1B). A representative example of discriminatory peaks presented in Fig 1C proved that the signal intensity of the peak increased with the prolonged time of infection. Another representative example of discriminatory peaks presented

in Fig 1D proved that the signal intensity of the peak increased in all different groups regardless of the parasite burden.

Detection and validation of SELDI peaks

BPS™ was then used to analyze the peak intensities by generating classification trees from all of the SELDI data. For example, Fig 2 shows a candidate diagnostic algorithm for acute infection based upon the IMAC array biomarker of 46 kDa. This single biomarker decision tree achieved 100% sensitivity and 100% specificity.

Biomarker identification

The identification of candidate biomarkers is crucial to their translation into useful clinical and scientific 'tools'. Unfortunately, the SELDI-TOF-MS platform does not permit peptide/protein identification, yielding only approximate molecular weight 'peaks'. To identify these peaks we used two different approaches: peptide 'fingerprinting' with MALDI-TOF and peptide sequencing with Orbitrap.

MALDI-TOF MS

We next used the MALDI-TOF for MS analysis of 1-dimensional SDS-PAGE gel slices of pooled sera from each group at different time points: 3, 6, and 12 weeks post infection. This approach generated eight potential biomarkers, all of host origin. Among these candidate biomarkers, serotransferrin and alpha 1-antitrypsin (AIAT) were the most convincing, as the former was identified with 12 peptides and the latter was identified with four peptides. These data suggest that these two proteins are present in infected mouse serum at relatively high abundance (Table 1).

Velos Orbitrap

The Orbitrap analysis was performed with mouse sera from acute and chronic stages of infection to pursue parasite antigens as promising candidates. Here we provide, for the first time, the identity of the set of schistosomal proteins detected from mouse serum by Velos Orbitrap mass spectrometry that show great promise as potential biomarkers. Scaffold revealed 597 and 454 proteins from samples of the acute and chronic mice sera, respectively. This number was reduced to 200 and 105 proteins by requiring 95% probability and the presence of at least one identified peptide (Supplemental Tables 1 and 2 in Appendex at the end). Scaffold also revealed 654 and 774 host proteins from the acute and chronic mice sera, respectively. As previously outlined, this number was also reduced significantly to 296 and 220 proteins by imposing the same conditions for acceptance

These parasite-origin proteins may reflect proteins released by damage to the tegument, or secretory/excretory products released by worms into mouse serum. It has been suggested that confidence in protein identification is increased by replication and independent identification of the same proteins (Cargile et al., 2004; Moore et al., 2002). In order to increase confidence in sensitive Orbitrap data, the Orbitrap data sets have been recorded in and compared (in replicated three experiments). Confidence also increases with the number of peptides identified from each protein (Cargile et al., 2004; Moore et al., 2002). Therefore, we filtered the results by Validation Category of Spectrum Mill Software using number of identified spectra, distinct peptide numbers, % amino acid (%AA) coverage, and total protein spectral intensity. Using Spectrum Mill Software, we brought the number of parasite-origin protein down to 28 proteins in both acute and chronic stages (Table 2). Among these 28 candidate biomarkers, actin was the most frequently-identified protein. Ryanodine receptor-related protein was identified with a distinct summed MS/MS search score of 15.36, %AA coverage of 0.1, and total protein spectral intensity

of 1.97E+09. Protocatechuate dioxygenase was also identified with a distinct summed MS/MS search score of 12.95, %AA coverage of 3.3, and total protein spectral intensity of 4.74E+06. Most proteins were identified with only one peptide however, which may be explained by the low concentrations of the parasite antigens compared to the host antigens in the serum of infected animals. Most of the protein identifications were based on single peptides putting their identification in doubt without additional confirmatory studies. When available, the classical approach for confirmation is the running of a Western blot. We performed Western blotting for GST (one of the proteins identified with only one peptide) and confirmed the MS identification (Fig 5).

Overlap between the three mass spectrometry platforms

Although SELDI-TOF provided proof-of-principle that serum from mice with acute and chronic infection had distinct protein patterns (from each other and from control animals), this approach could not identify the differentially expressed proteins. To overcome this limitation, we turned to the MALDI-TOF and Orbitrap platforms to identify the differentially expressed proteins. In our hands, MALDI-TOF had relatively limited sensitivity, identifing only eight host proteins over-expressed in the infected mice (haemoglobin beta, apolipoprotein A-I, serotransferrin precursor, hemopexin, serum albumin precursor, apolipoprotein A-IV precursor, alpha-1-antitrypsin, and beta-globin). All eight were also identified as up-regulated in infection by Orbitrap (100% probability) and all but hemopexin were reflected in corresponding SELDI peaks (±5% mass range) when the SELDI database was queried. Of the 66 candidate biomarker peaks identified by SELDI as differentially-expressed (either up- or down-regulated), all but 20 (30.3%) were tentatively identified in the Orbitrap database (±5% mass range) (data not shown). The consistency with which these candidate biomarkers were identified across the three different

platforms strongly supports the validity of these observations. Overlap between the proteomic biomarkers on the three MS platforms are recorded in Table 3.

Western Blot

To confirm the accuracy of the MALDI and Orbitrap analyses, we performed Western blots for some of the identified proteins. Both A1AT (alpha 1-antitrypsin) and serotransferrin were differentially expressed between infected and control serum samples (Fig 3 & 4). For AIAT, the whole protein (~50 kDa) was present but the antibody recognized two bands; the upper band corresponding to full-length A1AT while the lower band (~25 kDa) is likely an A1AT fragmentas, as described previously (Zelvyte& Janciauskiene, 2002). Effects of native and cleaved forms of α1-antitrypsin on ME 1477 tumor cell functional activity. Cancer detection and prevention, 26(4), 256-265.**.** Western blotting also confirmed that transferrin is highly expressed in the sera of infected mice, the whole protein (~79 kDa) was detected but the antibody recognized two bands; the upper band at 79 kDa while the lower band at 50 kDa is likely a transferrin fragment.

Orbitrap data suggested the presence of both mouse (~24 kDa) and schistosome (~25 kDa) GST, which was confirmed by Western blot. The intact whole protein (molecular weight ~24 kDa) was present in control sera while, the antibody recognized two bands in acutely and chronically-infected animals; the upper band (molecular weight ~25 kDa) corresponds to the schistosome GST while the lower band (~24 kDa) likely corresponds to the mouse GST (Fig 5). Orbitrap data also suggested the presence of schistosome actin (42 kDa), which was confirmed by Western Blot. The antibody recognized one band (42 kDa) in the sera of control and infected mice, both in acute and chronic stages (Fig 6).

Discussion

In 2003, two large transcriptome databases that contained resources for peptide searches for *S. mansoni* and *S. japonicum* were published (Verjovski-Almeida et al., 2003; Hu et al., 2003). The publication of the *S. mansoni* genome provided approximately 17,250 full-length predicted genes (Wilson et al., 2007). These genomic tools provide invaluable resources for protein identification.

There is an urgent need to provide early detection techniques of schistosomiasis so that the infection can be eliminated before egg deposition and complications occur. Given that SELDI-TOF MS is relatively high-throughput, requires minute sample for analysis, we chose this technique as our 'first-line' approach for biomarker discovery. Using SELDI, we were able to identify several candidate biomarkers with promise as markers of early-stage infection. SELDI results also suggested that the presence of these biomarkers was an indicator of infection rather than the intensity of infection. While the SELDI platform provided 'proof-of-concept', these data were restricted to biomarker 'peaks' rather than identified proteins.

In order to identify some of the SELDI biomarker peaks, we exploited the MALDI-TOF and Velos Orbitrap platforms. Serum transferrin and glycoprotein A1AT were identified by MALDI and confirmed by Western blot. Transferrin levels were increased in both acute and chronic stages of disease. Transferrin is a 'negative' acute phase reactant that is synthesized in the liver as a glycoprotein and is involved in iron transport (Gomme, 2005). Serum levels are increased in alcoholic fatty liver disease (Potter, 1985) but decreased in hepatic diseases that suppress synthetic capacity (Otegbayo, 2005). In human and murine schistosomiasis, serum transferrin levels have varied substantially between studies. For example, our finding are consistent with those of Salawu and Arinola (2004) who reported increased serum levels in urinary schistosomiasis. Harvie et al. (2007) also reported increased liver transferrin levels in

C57BL/6 mice after 8 weeks of *S. mansoni* infection. These same authors found elevated serum transferrin in chronic hepatosplenic schistosomiasis in CBA/J mice and suggested that this protein might be potential biomarker for hepatosplenic disease [35]. In contrast, Saif et al. (1977), Arinola (2004) and Arinola & Salimonu (1998) have reported decreased serum transferrin levels in hepatosplenic and urinary schistosomiasis in a sample of patients in Egypt and Nigeria, respectively. Plasma transferrin levels tend to increase in patients suffering from iron deficiency anemia (Macedo & Sousa, 2008) and Mansour & Farid (1985) have shown an association between *S. mansoni* infection and anemia. Given these previous findings from different studies, we postulate that increased levels of transferrin could be a result of gastrointestinal blood loss and iron deficiency anemia that is associated with schistosomiasis.

Four A1AT peptides were identified using the MALDI platform, A1AT is a member of the serpin family (serine protease inhibitors) and acts as a trypsin inhibitor. It is synthesized as a single polypeptide chain of about 51 kDa mainly in the liver but also in macrophages and epithelial cells (Hiemstra, 2002). Deficiency of this protein is associated with many liver diseases such as neonatal hepatitis (Sharp et al., 1969), cirrhosis and hepatoma (Eriksson et al., 1986). Finally, this protein is considered a potential biomarker for hepatitis B virus infection (Xu-fei Tan et al., 2011). The serpin family has been reported to increase in inflammation (Gettins, 2002). In our murine schistosomiasis model, A1AT was increased in both the acute and chronic stages of the infection, both of which are associated with significant inflammatory responses.

Surprisingly, the Velos Orbitrap identified a large number of putative parasite-origin proteins in serum of infected mice. The large number of schistosome proteins in the serum from the *S. mansoni* infected mice may be attributable, in part, to our use of the serum from the later

time-points (6 and 12 weeks post-infection) in the Orbitrap analysis. This strategy may have allowed schistosome proteins to accumulate in the blood after being secreted, shed from live parasites, or released upon parasite destruction mediated by the host immune system (Francis & Bickle, 1992; Jankovic, et al., 1996; Mohamed, et al., 1998). Many of the schistosome proteins that we found in low concentrations in the serum of the infected mice are potentially interesting in understanding the biology of schistosomiasis. However, the more abundant proteins are likely to be the most useful for clinical applications. We therefore, sought to prioritize the more abundant proteins for further validation using Validation Category of Spectrum Mill Software. Some of these proteins were known to be present in the parasite tegument, including (a) actin protein, which was the most abundantly identified protein with 7 identified peptides and 23.1% amino acid coverage; (b) tegumental protein, with 1 identified peptides and 3.2% amino acid coverage and (c) a 14 KDa calcium-binding protein of unknown function. The tegument of *S. mansoni* is limited by two lipid membrane bilayers that contain several spines (Morris & Threadgold, 1968; Wilson & Barnes, 1974). These spines are composed mainly of actin bundles (Cohen et al., 1982). Using fluorescence microscopy, actin has been present on the surface of schistosomula (Davis et al., 1985). In addition, actin proved to be present in the muscle, tegumental tubercles and spines of male and female adult parasites using immunofluorescence (MacGregor & Shore, 1990). Actin has been implicated in the maintenance of the integrity of the tegument of the adult worm, either associated with the spines, or free in the cytoplasm (McCormick & Damian, 1987). Our finding of actin in the serum of the infected mice might be the result of shedding from the schistosomula released from dying worms or damaged to the tegumental cell layer.

We also identified released enzymes such as GST that play a crucial role in defence against oxidative stress (Trotteinet et al., 1990). Heat shock protein (HSP60) was also identified as an abundant protein in this proteomic analysis. By looking for commercial antibodies for one of these potential biomarkers we found one against *S. japonicum* GST. The glutathione S-transferase (GST) enzyme family uses glutathione in reactions contributing to the detoxification of endogenous and exogenous toxins (Hayes & Pulford, 1995). Increased levels of host GST have been reported in hepatocellular carcinoma (Hayes, et al., 1991) and schistosomiasis (Harvie, et al., 2007). However, Manivannan, et al. (2010) reported decreased levels of GST in *S. mansoni* infected mice. In our study, we found overexpression of GST in acute and chronic stages of *S. mansoni* infected mice. The overexpressed GST reacted with anti-GST antibody of *S. japonicum*. This suggests that the GST originated from the parasite as identified by Velos Orbitrap. Therefore, GST can be considered as an infection-associated antigen and its overexpression may be considered as a potential serum biomarker for the early diagnosis of *S. mansoni*.

Summary and conclusion:

Large numbers of candidate host- and parasite-origin biomarkers for acute and chronic schistosomiasis in mice were identified using a combination of SELDI, MALDI and Orbitrap technologies. We found little overlap in the biomarkers identified (based on m/z); most appeared to be unique. These three platforms, therefore, performed in a complementary fashion in our biomarker discovery program. Our program revealed that serum protein profiles differ extensively between infected and uninfected mice and between the early, acute and chronic stages of infection in mice, offering a rich source of candidate biomarkers. If similar differences are identified in human disease, this approach may not only yield new diagnostic strategies but

may also give insights into parasite biology and point to novel targets for treatment or prevention.

FIGURES AND TABLES

Fig 1A. Scatterplot showing the discrimination of the early infection and control groups using m/z 3720 at F6ISL (**F**raction **6**, **IMAC** chip, **L**ow laser intensity). Blue dots: control. Red dotes: early stage of infection.

Fig 1B. Scatterplot showing the discrimination of the acute and chronic groups using m/z 13407.2 at F6CSH (**F**raction **6**, **CM10** chip, **H**igh laser intensity). Blue dots: acute. Red dotes: chronic.

Fig 1C: Example of a candidate biomarker increased in infected mice over time. Serum SELDI-TOF MS mass spectra obtained for F6ISL (**F**raction **6**, **IMAC** chip, **L**ow laser intensity) from infected mice (top 3 spectra) versus non-infected mice (bottom spectrum). A candidate biomarker as 7081 Da is gradually up-regulated from 3, to 6, to 12 weeks after infection (P value 0.006).

Fig 1D: Example of a candidate biomarker increased in infected mice regardless the parasite burden. Serum SELDI-TOF MS mass spectra obtained for F6CSL (**F**raction **6**, **CM10** chip, **L**ow laser intensity) from infected mice (bottom 4 spectra) versus non-infected mice (top spectrum). A candidate biomarker as 5566.31 Da is up regulated in infected mice dependent on the infection rather than the dose of infected agents (P value 0.006).

Fig 1A

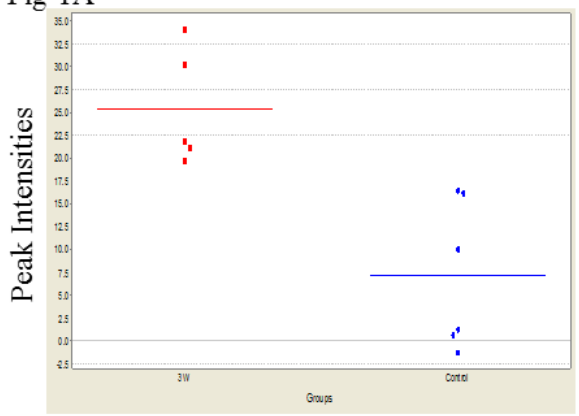

Fig 1B

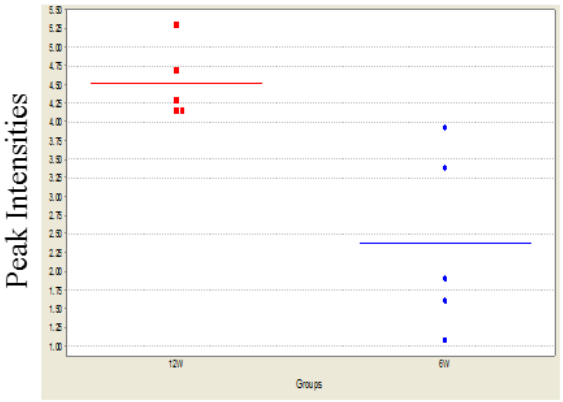

Fig 1C

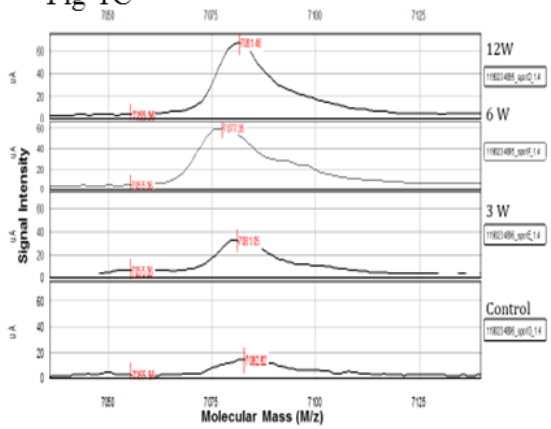

Fig 1D

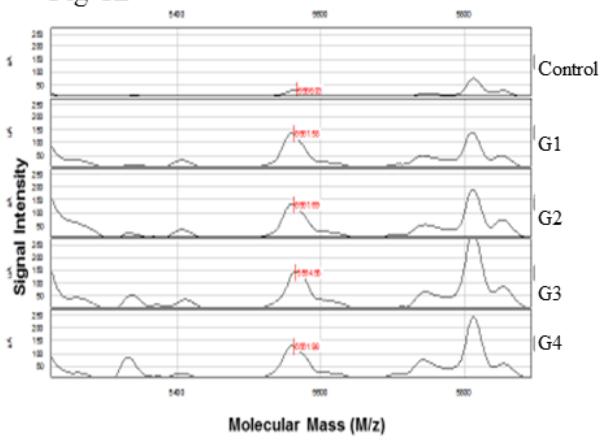

Fig 2: Biomarker pattern software based on CART analysis was used to generate candidate diagnostic algorithms. An example of decision tree classification is shown. In this algorithm, the intensities of the 46,106 Da biomarker establish the splitting rules. The samples have an intensity of ≤0.423 are placed in the left daughter node, and samples that have an intensity of ≥0.423 go to the right daughter node. Terminal red boxes = uninfected; blue boxes = acute infection.

Fig 2

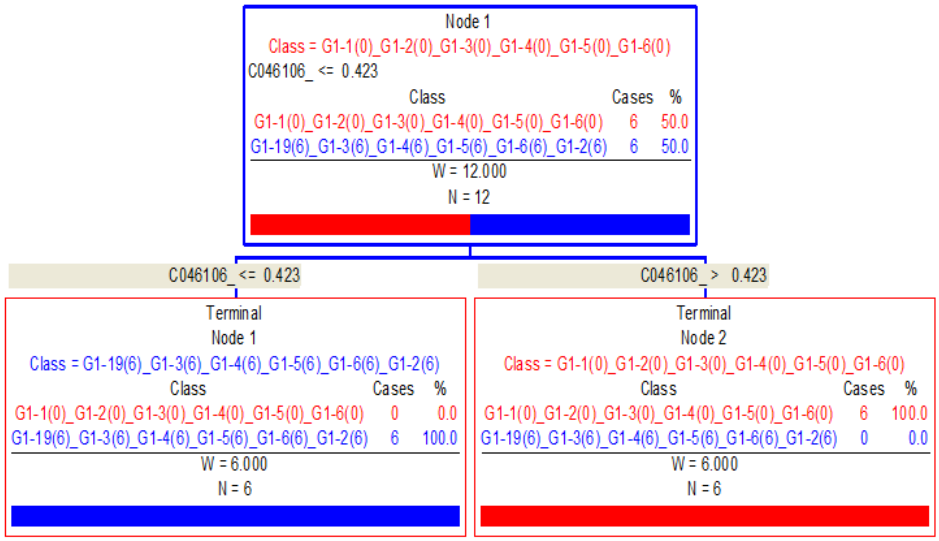

Fig 3: Immunologic validation of A1AT as a candidate biomarker I A) Representative Western blot of A1AT in pooled sera from *S. mansoni*-infected mice (6 and 12 weeks post-infection) and controls. B) Ponceau stained gel and C) Western blot of GAPDH served as a loading controls. D) Relative density of A1AT proteins levels normalized to GAPDH.

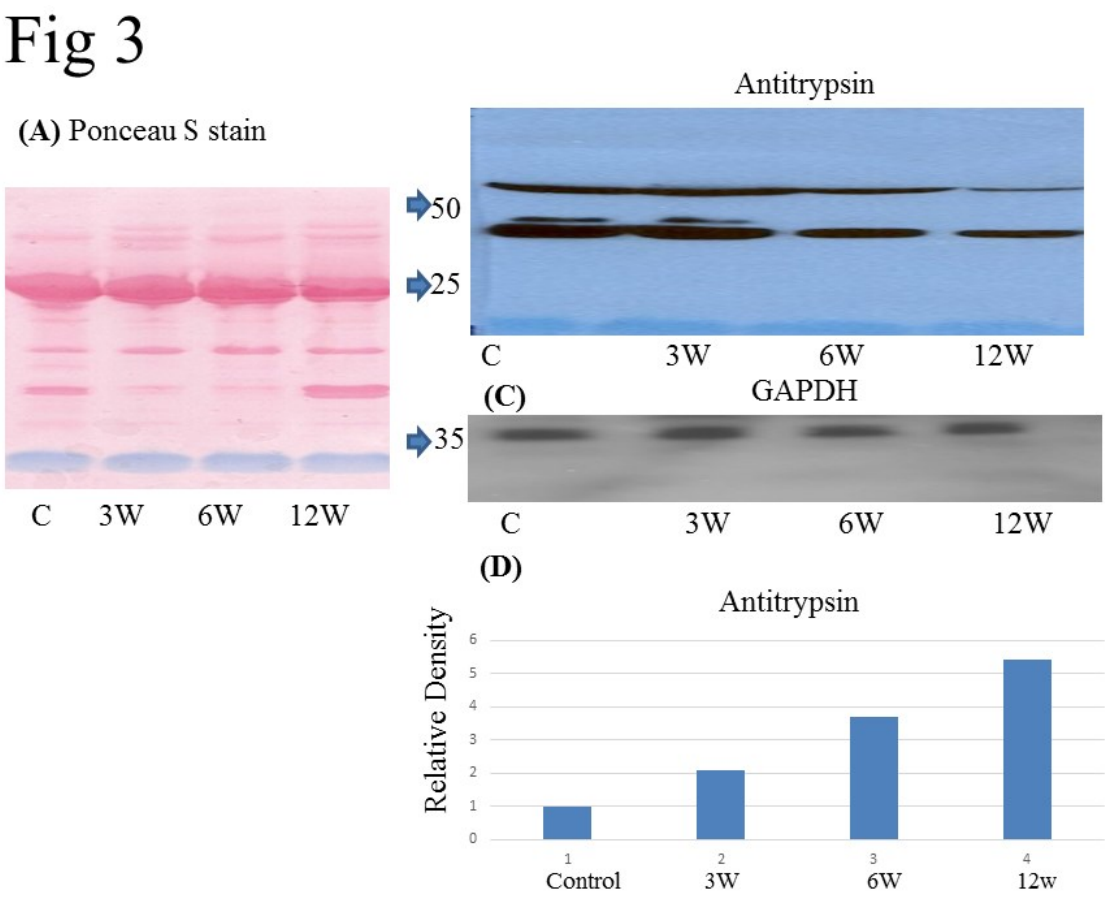

Fig 4: Immunologic validation of transferrin as a candidate biomarker I A) Representative Western blot of transferrin in pooled sera from *S. mansoni*-infected mice (6 and 12 weeks post-infection) and controls. B) Ponceau stained gel and C) Western blot of GAPDH served as a loading controls. D) Relative density of transferrin proteins levels normalized to GAPDH.

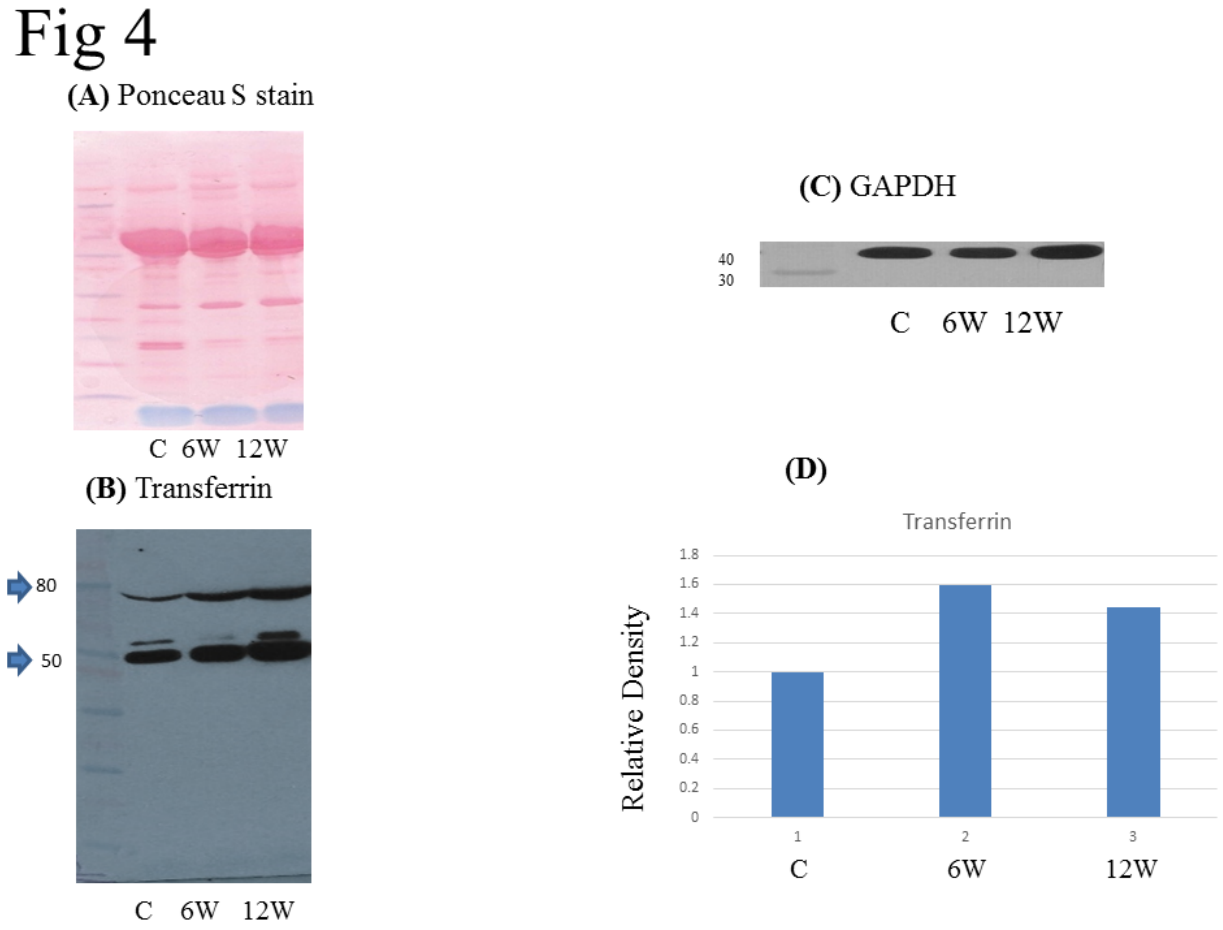

Fig 5: Immunologic validation of GST as a candidate biomarker I A) Representative Western blot of transferrin in pooled sera from *S. mansoni*-infected mice (3, 6 and 12 weeks post-infection as well as sample from 6 weeks post infection from different study) and controls. B) Western blot of GAPDH served as a loading controls. C) Relative density of GST proteins levels normalized to GAPDH.

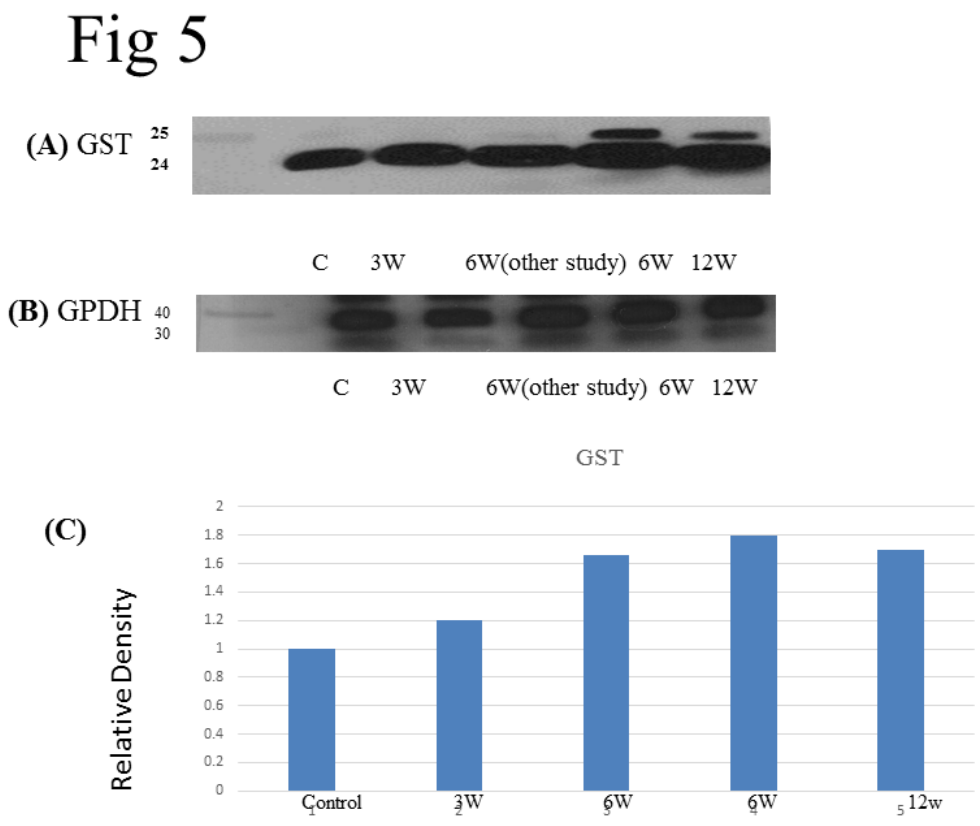

Fig 6: Immunologic validation of actin as a candidate biomarker I A) Representative Western blot of actin in pooled sera from *S. mansoni*-infected mice (6 and 12 weeks post-infection) and controls. B) Ponceau stained gel and C) Western blot of GAPDH served as a loading controls. D) Relative density of actin proteins levels normalized to GAPDH.

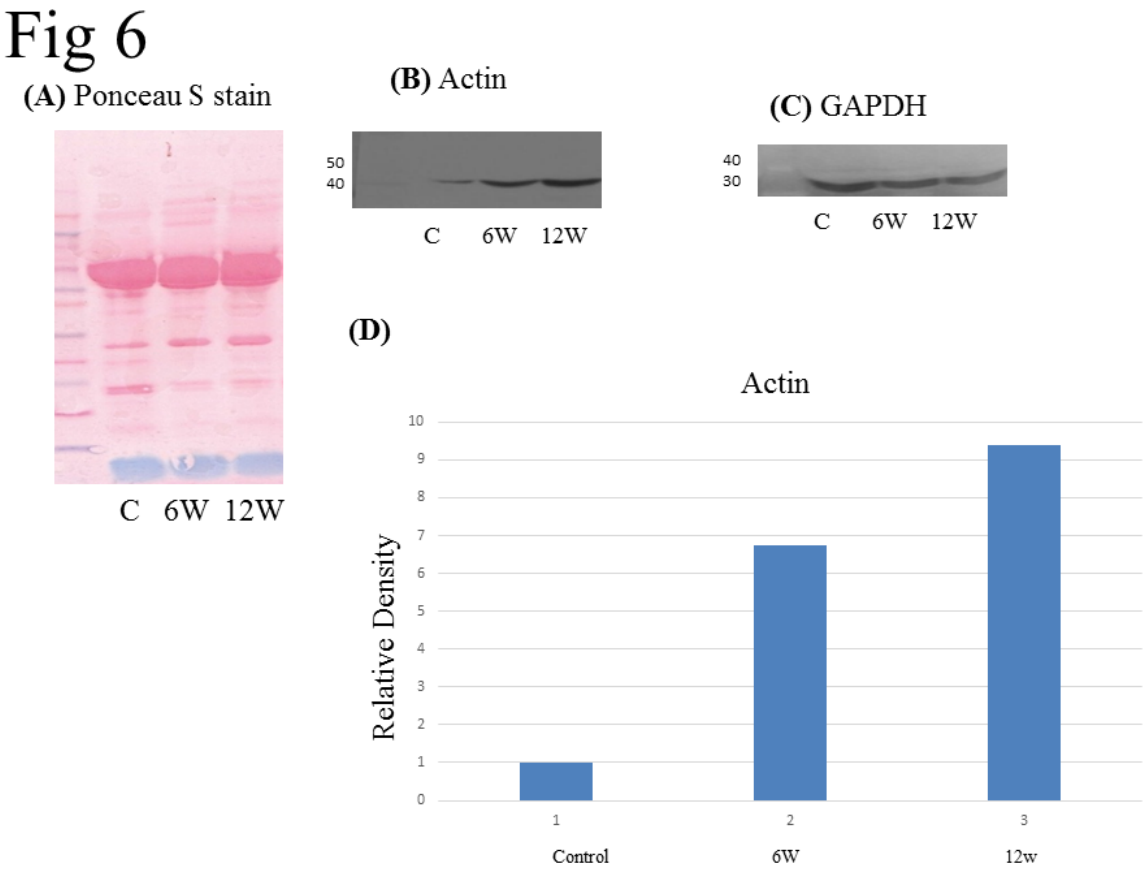

Table 1: Eight proteins were increased in the sera of mice during both the acute and chronic stages of schistosomiasis compared to controls.

N	Unused	Total	%Cov	%Cov(50)	%Cov(95)	Accession	Name	Species	Peptides (95%)
1	3.36	3.36	28.16	28.16	28.16	tr|E9Q223|E9Q223_MOUSE	Hemoglobin subunit beta-1	MOUSE	4
3	2.01	2.01	9.091	4.924	4.924	tr|Q8BPD5|Q8BPD5_MOUSE	Apoa1 protein	MOUSE	2
1	20	20	12.58	12.58	12.58	tr|E9Q035|E9Q035_MOUSE	Serotransferrin	MOUSE	12
3	2	2	5.217	5.217	5.217	sp|Q91X72|HEMO_MOUSE	Hemopexin	MOUSE	2
2	0	2	2.138	2.138	2.138	tr|Q546G4|Q546G4_MOUSE	Albumin 1	MOUSE	1
3	2.56	2.56	7.089	7.089	4.557	tr|Q9DBN0|Q9DBN0_MOUSE	Apolipoprotein A-IV	MOUSE	1
3	2	2	5.327	5.327	5.327	tr|Q91X22|Q91X22_MOUSE	Alpha-1-antitrypsin	MOUSE	4
1	2	2	6.849	6.849	6.849	tr|Q9QUN8|Q9QUN8_MOUSE	Beta-2-globin	MOUSE	4

N: The rank of the specified protein relative to all other proteins in the list of detected proteins.

Unused (ProtScore): A measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely "used" by higher scoring winning proteins.

Total (ProtScore): A measure of the total amount of evidence for a detected protein. The Total ProtScore is calculated using all of the peptides detected for the protein.

% Cov (Coverage): The percentage of matching amino acids from identified peptides having confidence greater than 0 divided by the total number of amino acids in the sequence.

% Cov (50): The percentage of matching amino acids from identified peptides having confidence greater than or equal to 50% divided by the total number of amino acids in the sequence.

% Cov (95): The percentage of matching amino acids from identified peptides having confidence greater than or equal to 95% divided by the total number of amino acids in the sequence.

Accession #: The accession number for the protein.

Peptides (95%): The number of distinct peptides having at least 95% confidence.

Table 2: Shows these proteins ordered by Spectrum Mill; the higher abundant identified proteins.

Spectra	Distinct peptide	Distinct Summed MS/MS Search score	% AA Coverage	Total Protein Spectral intensity	Database Accession number	Protein Name
223	10	157.6	14.1	6.00E+11	15808978	albumin precursor
11	7	108.9	23.1	9.59E+08	2.56E+08	Actin
13	1	15.36	0.1	1.97E+09	2.56E+08	ryanodine receptor related
1	1	13.17	5.9	6.85E+06	2.56E+08	rap1 and
1	1	12.95	3.3	4.74E+06	2.56E+08	protocatechuate dioxygenase
1	1	12.67	10.1	1.88E+06	2.83E+08	MHC class I antigen
1	1	12.57	24.4	1.37E+09	2.56E+08	hypothetical protein
1	1	12.41	2.1	2.07E+08	2.56E+08	hypothetical protein
1	1	11.74	4.4	1.32E+08	76152891	SJCHGC08921 protein
3	1	11.71	2	6.13E+08	2.56E+08	camp-response element binding protein-related
1	1	11.59	5	7.07E+06	2.56E+08	elongation factor 1-alpha (ef-1-alpha)
1	1	11.25	0.8	7.41E+06	2.56E+08	hypothetical protein
1	1	11.05	6.3	3.67E+0	2.56E+0	mitochondrial phosphate carrier

				7	8	protein
1	1	10.7	0.7	8.17E+06	2.56E+08	lyst-interacting protein
1	1	10.66	3.7	3.52E+08	76155947	SJCHGC04200 protein
1	1	10.53	2.3	9.41E+06	2.56E+08	hypothetical protein
1	1	10.51	3.7	5.85E+06	2.56E+08	glycerol-3-phosphate dehydrogenase
1	1	10.49	5.6	2.84E+09	76152647	SJCHGC05123 protein
1	1	10.36	7.9	3.56E+07	76162212	SJCHGC01996 protein
1	1	10.35	3.1	1.36E+07	2.56E+08	hypothetical protein
1	1	10.29	6.8	6.26E+07	2.57E+08	Tropomyosin-2
1	1	10.28	0.4	8.04E+07	2.56E+08	hypothetical protein
1	1	10.26	3.4	3.38E+06	2.56E+08	heat shock protein
1	1	10.24	0.7	2.82E+07	2.56E+08	protein kinase
1	1	10.19	10.7	1.46E+08	76152933	SJCHGC03943 protein
1	1	10.11	1.9	9.85E+06	2.56E+08	myotubularin-related protein
1	1	10.02	0.2	5.19E+08	2.56E+08	hypothetical protein
1	1	10.01	3.2	2.72E+06	2.56E+08	tegumental protein

Table 3: Overlap between the three methods (SELDI, MALDI, and Orbitrap). This table shows the number of peaks detected in serum by MALDI and the overlap between them and the similar peaks detected by the other two methods Orbitrap and SELDI.

MALDI	Orbitrap		Orbitrap (acute stage)		Orbitrap (chronic stage)		SELDI	
Name	Accession N.	molecular weight	Peptides (95%)	AA coverage	Peptides (95%)	AA coverage	molecular weight	M/Z Average
Hemoglobin subunit beta-1	1183933	16 kDa	6	89%	1	96%	16297.84	F6ISL
Apoa1 protein	231557	31 kDa	27	77%	23	71%	34996.4	F6ISH
Serotransferrin	20330802	77 kDa	65	73%	52	83%	82364	F6CSH
Hemopexin	15030012	51 kDa	28	64%	23	61%		
Albumin 1	163310765	69 kDa	65	88%	58	85%	64508.29	F1ISH
Apolipoprotein A-IV	110347473	45 kDa	26	74%	20	63%	46278.31	F6ISH
Alpha-1-antitrypsin	6678085	46 kDa	4	47%	21	60%	46573.27	F1ISH
Beta-2-globin	156257619	16 kDa	3	93%	2	86%	16297.84	F6ISL

References

Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ, 2008. The use of total protein stains as

loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. *Journal of Neuroscience Methods 172*(2):250-4.

Arinola, O. G., & Salimonu, L. S. (1998). Humoral immunity in Nigerians with Schistosoma haematobium infection. *West African Journal of Medicine, 18*(4), 275-280.

Arinola, O. G. (2004). Metal binding acute phase proteins and trace elements in Nigerian children

with urinary schistosomiasis. *Biokemistri 16*(1), 23-27.

Bergquist, R., Johansen, M. V., & Utzinger, J. (2009). Diagnostic dilemmas in helminthology:what

tools to use and when?. *Trends in Parasitology, 25*(4), 151-156.

Cargile, B. J., Bundy, J. L., & Stephenson, J. L. (2004). Potential for false positive identifications from large databases through tandem mass spectrometry. *Journal of Proteome Research, 3*(5), 1082-1085.

Chen, L., Ho, D. W., Lee, N. P., Sun, S., Lam, B., Wong, K. F., ... & Luk, J. M. (2010). Enhanced detection of early hepatocellular carcinoma by serum SELDI-TOF proteomic signature combined with alpha-fetoprotein marker. *Annals of Surgical Oncology, 17*(9), 2518-2525.

Cohen, M., Yossef, R., Erez, T., Kugel, A., Welt, M., Karpasas, M. M., .. & Porgador, A. (2011). Serum apolipoproteins CI and C-III are reduced in stomach cancer patients: results from MALDI-based peptidome and immuno-based clinical assays. *PloS one, 6*(1), e14540.

Davis, A. H., Blanton, R., & Klich, P. (1985). Stage and sex specific differences in actin gene expression in Schistosoma mansoni. *Molecular and Biochemical Parasitology, 17*(3), 289-298.

Deelder, A. M., Qian, Z. L., Kremsner, P. G., Acosta, L., Rabello, A. L., Enyong, P., ... & Rotmans,

J. P. (1993). Quantitative diagnosis of Schistosoma infections by measurement of circulating antigens in serum and urine. *Tropical and Geographical Medicine, 46*(4), 233-238.

Doenhoff, M. J., Chiodini, P. L., & Hamilton, J. V. (2004). Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies?. *Trends in Parasitology, 20*(1), 35-39.

Eriksson, S., Carlson, J., & Velez, R. (1986). Risk of cirrhosis and primary liver cancer in alpha1-antitrypsin deficiency. *New England Journal of Medicine, 314*(12), 736-739.

Espírito-Santo, M. C. C., Alvarado-Mora, M. V., Pinto, P. L. S., Sanchez, M. C. A., Dias-Neto, E., Castilho, V. L. P., ... & Carrilho, F. J. (2015). Comparative Study of the Accuracy of Different Techniques for the Laboratory Diagnosis of Schistosomiasis Mansoni in Areas of Low Endemicity in Barra Mansa City, Rio de Janeiro State, Brazil. *BioMed Research International,* 2015.

Francis, P., & Bickle, Q., (1992). Cloning of a 21.7-kDa vaccine-dominant antigengene of Schistosoma mansoni reveals an EF hand-like motif. *Mol BiochemParasitol 50*(2), 215–224.

Gettins, P. G. (2002). Serpin structure, mechanism, and function. *Chemical Reviews, 102*(12), 4751-4804.

Gomme, P. T., McCann, K. B., & Bertolini, J. (2005). Transferrin: structure, function and potential

therapeutic actions. *Drug Discovery Today, 10*(4), 267-273

Greter, H., Krauth, S. J., Ngandolo, B. N., Alfaroukh, I. O., Zinsstag, J., & Utzinger, J. (2015). Validation of a Point-of-Care Circulating Cathodic Antigen Urine Cassette Test for Schistosoma mansoni Diagnosis in the Sahel, and Potential Cross-Reaction with Pregnancy. The American Journal of Tropical Medicine and Hygiene, 15-0577.

Gryseels B, Polman K, Clerinx J, Kestens L. Human schistosomiasis. *The Lancet,368*(9541):1106-18.

Harvie, M., Jordan, T. W., & La Flamme, A. C. (2007). Differential liver protein expression during schistosomiasis. *Infection and Immunity, 75*(2), 736-744.

Hayes, J. D., & Pulford, D. J. (1995). The Glutathione S-Transferase Supergene Family:

Regulation of GST and the Contribution of the lsoenzymes to Cancer Chemoprotection and Drug Resistance Part I. *Critical Reviews in Biochemistry and Molecular Biology, 30*(6), 445-520.

Hayes, P. C., May, L., Hayes, J. D., & Harrison, D. J. (1991). Glutathione S-transferases in human liver cancer. *Gut, 32*(12), 1546-1549.

Hiemstra, P. S. (2002). Novel roles of protease inhibitors in infection and inflammation. *Biochemical Society Transactions, 30*(2), 116-119.

Hotez, P. J., & Fenwick, A. (2009). Schistosomiasis in Africa: an emerging tragedy in our new global health decade. *PLoS Neglected Tropical Diseases, 3*(9), e485.

Hu, W., Yan, Q., Shen, D. K., Liu, F., Zhu, Z. D., Song, H. D. Xu, X.R. Wang, Z.J. Rong, Y.P. Hutchens, T. W., & Yip, T. T. (1993). New desorption strategies for the mass spectrometric analysis of macromolecules. *Rapid Communications in Mass Spectrometry, 7*(7), 576-580.

Jankovic, D., Aslund, L., Oswald, I. P., Caspar, P., Champion, C., Pearce, E., ... & James, S. L. (1996). Calpain is the target antigen of a Th1 clone that transfers protective immunity against Schistosoma mansoni. *The Journal of Immunology, 157*(2), 806-814.

Jordan, P., & Webbe, G. (1969). Human schistosomiasis. Human schistosomiasis.

Lin, Q., Peng, Q., Yao, F., Pan, X. F., Xiong, L. W., Wang, Y., & Wang, J. C. (2012). A classification method based on principal components of SELDI spectra to diagnose of lung adenocarcinoma. *PloS One, 7*(3), e34457.

Liu, N. K., & Xu, X. M. (2006). β-Tubulin is a more suitable internal control than β-actin in Western blot analysis of spinal cord tissues after traumatic injury. *Journal of Neurotrauma, 23*(12), 1794-1801.

Lovric, J. (2011). Introducing Proteomics: From Concepts to Sample Separation. *Mass Spectrometry and Data Analysis, Wiley, Hoboken, NJ*.

Macedo, M. F., & Sousa, M. D. (2008). Transferrin and the transferrin receptor: of magic bullets and other concerns. *Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy), 7*(1), 41-52.

MacGregor, A. N., & Shore, S. J. (1990). Immunocytochemistry of cytoskeletal proteins in adult Schistosoma mansoni. *International Journal for Parasitology, 20*(3), 279-284.

Manivannan, B., Rawson, P., Jordan, T. W., Secor, W. E., & La Flamme, A. C. (2010).

Differential patterns of liver proteins in experimental murine hepatosplenic schistosomiasis. *Infection and Immunity, 78*(2), 618-628.

Mansour, M. M., Francis, W. M., & Farid, Z. O. H. E. I. R. (1985). Prevalence of latent iron deficiency in patients with chronic S. mansoni infection. *Tropical and Geographical Medicine, 37*(2), 124-128.

McCormick, S. L., & Damian, R. T. (1987). Haptenation of adult Schistosoma mansoni and assessment of humorally mediated damage in vitro. *The Journal of Parasitology*, 130-143.

Mohamed, M. M., Shalaby, K. A., LoVerde, P. T., & Karim, A. M. (1998). Characterization of Sm20. 8, a member of a family of schistosome tegumental antigens. *Molecular and Biochemical Parasitology, 96*(1), 15-25.

Moore, R. E., Young, M. K., & Lee, T. D. (2002). Qscore: an algorithm for evaluating SEQUEST database search results. *Journal of the American Society for Mass Spectrometry, 13*(4), 378-386.

Morris GP, Threadgold LT. Ultrastructure of the tegument of adult Schistosoma mansoni. *The Journal of Parasitology*,*1* :15-27.

Ndao M, Spithill TW, Caffrey R, Li H, Podust VN, Perichon R, Santamaria C, Ache A, Duncan M, Powell MR, Ward BJ, 2010. Identification of novel diagnostic serum biomarkers for Chagas' disease in asymptomatic subjects by mass spectrometric profiling. *Journal of Clinical Microbiology, 48(*4), 1139-1149.

Otegbayo, J. A., Arinola, O. G., Aje, A., Oluwasola, O. A., Okiwelu, O. H., & Salimonu, L. S. (2005). Usefulness of acute phase proteins for monitoring development of hepatocellular carcinoma in hepatitis B virus carriers. *West African Journal of Medicine, 24*(2), 124-127.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research, 29*(9), e45-e45.

Pontes, L. A., Dias-Neto, E., & Rabello, A. (2002). Detection by polymerase chain reaction of Schistosoma mansoni DNA in human serum and feces. *The American Journal of Tropical Medicine and Hygiene, 66*(2), 157-162.

Potter, B. J., Chapman, R. W., Nunes, R. M., Sorrentino, D., & Sherlock, S. (1985). Transferrin metabolism in alcoholic liver disease. *Hepatology, 5*(5), 714-721.

Rioux, M. C., Carmona, C., Acosta, D., Ward, B., Ndao, M., Gibbs, B. F., Bennett, H. P. & Spithill, T. W. (2008). Discovery and validation of serum biomarkers expressed over the first twelve weeks of Fasciola hepatica infection in sheep. *International Journal for Parasitology, 38*(1), 123-136.

Saif, M., Tawfik, J., Ali, M. A., El-Mahrouky, S. F., Galil, N., & Galal, M. N. (1976). Serum transferrin, albumin and IgG levels in hepatosplenic bilharziasis in Egypt. *The Journal of the Egyptian Medical Association, 60*(9-12), 731-743.

Salawu, L., & Arinola, O. G. (2004). Acute phase proteins in pregnant women with urinary schistosomiasis in Ilie Village, Osun state, Nigeria. *African Journal of Biomedical Research, 7*(3).

Santamaria, C., Chatelain, E., Jackson, Y., Miao, Q., Ward, B. J., Chappuis, F., & Ndao, M. (2014). Serum biomarkers predictive of cure in Chagas disease patients after nifurtimox treatment. *BMC Infectious Diseases, 14*(1), 1.

Sharp, H. L., Bridges, R. A., Krivit, W. I. L. L. I. A. M., & Freier, E. F. (1969). Cirrhosis associated with alpha-1-antitrypsin deficiency: a previously unrecognized inherited disorder. *J Lab Clin Med, 73*(6), 934-939.

Steinberg, D., & Colla, P. (1995). CART: tree-structured non-parametric data analysis. San Diego, CA: Salford Systems.

Tamura, M., Mochizuki, N., Nagatomi, Y., Harayama, K., Toriba, A., & Hayakawa, K. (2015). A Method for Simultaneous Determination of 20 Fusarium Toxins in Cereals by High-Resolution Liquid Chromatography-Orbitrap Mass Spectrometry with a Pentafluorophenyl Column. *Toxins, 7*(5), 1664-1682.

Tan, X. F., Wu, S. S., Li, S. P., Chen, Z., & Chen, F. (2011). Alpha-1 antitrypsin is a potential biomarker for hepatitis B. *Virology Journal, 8*(1), 274.

Trottein, F., Kieny, M. P., Verwaerde, C., Torpier, G., Pierce, R. J., Balloul, J. M., ... & Capron, A. (1990). Molecular cloning and tissue distribution of a 26-kilodalton Schistosoma mansoni glutathione S-transferase. *Molecular and Biochemical Parasitology, 41*(1), 35-44.

Verjovski-Almeida, S., DeMarco, R., Martins, E. A., Guimarães, P. E., Ojopi, E. P., Paquola, A.C., Piazza, J.P. Nishiyama, M.Y. Kitajima, J.P. Adamson, R.E. Ashton, P.D. Bonaldo, M.F. Coulson, P.S. Dillon, G.P. Farias, L.P. Gregorio, S.P. Ho, P.L. Leite, R.A.

Malaquias, L.C. Marques, R.C. Miyasato, P.A. Nascimento, A.L. Ohlweiler, F.P. Reis, E.M. Ribeiro, M.A. Sa, R.G. Stukart, G.C. Soares, M.B. Gargioni, C. Kawano, T. Rodrigues, V. Madeira, A.M. Wilson, R.A. Menck, C.F. Setubal, J.C. Leite, L.C. & Dias-Neto, E. (2003). Transcriptome analysis of the acoelomate human parasite Schistosoma mansoni. *Nature Genetics, 35*(2), 148-157.

Welinder C, & Ekblad L, 2012. Coomassie staining as loading control in Western blot analysis. *J Proteome Res, 10* (3),1416–1419.

Wilson, R. A., Ashton, P. D., Braschi, S., Dillon, G. P., Berriman, M., & Ivens, A. (2007). 'Oming in on schistosomes: prospects and limitations for post-genomics. *Trends in Parasitology, 23*(1), 14-20.

Wilson, R. A., & Barnes, P. E. (1974). The tegument of Schistosoma mansoni: observations on the formation, structure and composition of cytoplasmic inclusions in relation to tegument function. *Parasitology, 68*(02), 239-258.

Zelvyte, I., Sjögren, H. O., & Janciauskiene, S. (2002). Effects of native and cleaved forms of α1-antitrypsin on ME 1477 tumor cell functional activity. Cancer detection and prevention, 26(4), 256-265.

CONNECTING STATEMENT I

In the previous manuscript, we reported the presence of several biomarkers in the sera of *S. mansoni* infected mice sera in comparison to control using several different proteomic platforms. In the next manuscript, we studied carbonic anhydrase 1 in more detail as one of the most interesting of the candidate serum biomarkers in *S. mansoni* infected mice and humans.

CHAPTER III

Serum Carbonic anhydrase 1 is a biomarker for diagnosis of human *Schistosoma mansoni* infection

Kardoush MI[1, 2, 3], Ward BJ[2, 4], Ndao M[2, 4*]

Submitted and accepted with minor revision for publication in American Journal of Tropical Medicine & Hygiene

Affiliations:

[1]Institute of Parasitology, McGill University, Montreal, Quebec, Canada

[2] National Reference Centre for Parasitology, Research Institute of the McGill University Health Centre, Montreal General Hospital, Montreal, Quebec, Canada

[3]Department of Parasitology, Faculty of medicine, Benha University, Benha, Qalubia, Egypt

[4]JD MacLean Tropical Diseases Centre, Research Institute of the McGill University Health Centre, Montreal General Hospital, Montreal, Quebec, Canada

* Corresponding Author

Momar Ndao

Research Institute of the McGill University Health Center
Glen site – EM3-3244
1001 Décarie Blvd,
Montréal, QC,
H4A 3J1
Telephone: 514-934-1934 x44537
FAX: 514-934-8224
Email: momar.ndao@mcgill.ca

Keywords: *Schistosoma mansoni*, diagnostics, proteomics, biomarkers, carbonic anhydrase 1

Word Counts: Abstract: 199

Text: 3678

5 Figures/ 3 Tables

Abstract

Schistosoma mansoni is a major public health threat in many parts of the world. The current diagnostic tests for schistosomiasis are sub-optimal, particularly early in infection, when the parasite burden is low and with reinfection following treatment. We sought to identify novel biomarkers of active infection by studying serum proteins in a mouse model of schistosomiasis followed by confirmation in chronically-infected patients. Acute (six weeks) and chronic (12 weeks) sera from *S. mansoni*-infected C57Bl/6 mice as well as sera from chronically-infected patients were assessed using two proteomic platforms: surface-enhanced, laser-desorption and ionization, time-of-flight mass spectrometry (SELDI TOF MS) and Velos Orbitrap MS. Several candidate biomarkers were further evaluated by Western blot and/or ELISA. Among the most promising was carbonic anhydrase1 (CA1); a host protein found primarily in red blood cells and enterocytes that proved to be a negative biomarker for schistosomiasis in both mouse and human samples. Reduced serum CA-1 levels were confirmed by both Western blot (murine and human: both $p<0.001$) and ELISA (human: $p<0.01$). Western blots of serial mouse sera revealed a progressive reduction in serum CA1 levels over the 12-week infection period. CA1 is a promising negative serum biomarker for the diagnosis of *S. mansoni* infection.

Introduction:

Schistosomiasis affects ~200 million people in more than 70 countries and causes at least 200,000 deaths/year. More than 600 million people are at risk of acquiring this disease. Diagnosis typically depends on the detection of eggs that first appear in stool or urine between 4-6 weeks post-infection. During the late stages of the infection, which are associated with liver fibrosis and other complications, eggs can be difficult to detect and greater emphasis is placed on serologic testing. Current immunoassays have major limitations however, including the inability to distinguish active from resolved infection (ie: post-treatment), poor specificity due to the use of crude antigens and low sensitivity in patients with light infections (Elhag et al., 2011; Kinkel et al., 2012). Recent infections can also be missed due to a seronegative 'window' that typically lasts 4-12 weeks post-exposure (Hipgrave et al., 1997; Visser, et al., 1995; Whitty et al., 2000). Some patients can take up to 6 months to become seropositive, however (Golledge, 1995; Jones et al., 1992; Jordan & Webbe, 1969; Doenhoff, 2004).

Mouse models of *S. mansoni* infection vary widely with some inbred strains showing pathology similar to human disease (e.g.: C57BL/6) while others develop more severe manifestations (e.g.: CBA) (Bica et al., 2000; Idle et al., 2001; Pearce & MacDonald, 2002 Rashika et al., 2006). Regardless of the model used, the acute stage of murine schistosomiasis typically starts within 6-8 weeks of infection when active granulomas begin to form around eggs deposited in the colonic wall or swept up the portal circulation into the liver. At 12-14 weeks post-infection, mice enter the chronic stage and granuloma size tends to decrease (Byram & Lichtenberg, 1977; Doenhoff, et al., 1979; Byram, et al., 1979; Amiri, et al., 1992). Host granuloma formation and fibrosis associated with parasite eggs are the main pathologies associated with chronic schistosomiasis. These changes occur primarily in the liver and intestines

in the case of *S. mansoni* infection (Bica, et al., 2000; Pearce & MacDonald, 2002). *S. mansoni* infection in humans ranges from asymptomatic to severe hepatosplenic fibrosis with portal hypertension, gastrointestinal hemorrhage and death (Bica, et al., 2000; Pearce & MacDonald, 2002). Because of the similarities between murine and human disease manifestations, we used the C57BL/6 model to study protein profiles after *S. mansoni* infection to look for candidate biomarkers.

We followed a structured approach to biomarker discovery beginning with high throughput SELDI TOF MS analysis of mouse sera followed by Orbitrap Velos MS microsequencing and confirmatory immunologic testing (Western blot, ELISA). This approach revealed serum carbonic anhydrase 1 (CA-1) as a powerful biomarker in both acute and chronic infection in the mouse model and in chronically-infected patients.

Materials and Methods:

Infection protocol

S. mansoni -infected *Biomphalaria glabrata* snails were obtained from the Biomedical Research Institute (Bethesda, MD). Cercariae were collected 35–45 days post-infection by exposing the snails to continuous light for 2 hours at room temperature (RT) (Lewis et al., 1986). Twenty-six, six week-old female C57Bl/6 mice were purchased from Charles River (St. Constant, Québec). Twenty of the mice were infected by tail penetration (200 cercariae) as previously described (Lewis et al., 1986) and six uninfected animals served as controls. All animal experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and with approval of the Animal Care Committee of McGill University.

Murine and Human Serum Samples

At six weeks post-infection (acute phase), 15 of the infected mice and three control animals were scarified. At 12 weeks post-infection (chronic phase), five infected and three control mice were sacrificed. At sacrifice, blood was obtained by cardiac puncture, adult worms in the mesenteric veins were counted, and livers were collected for histology. Mouse sera were aliquoted and kept at −80°C until assays were performed. Human serum samples from a total of 25 patients (16 males and 9 females; age range 10-60 years) diagnosed with schistosomiasis were obtained from the National Reference Centre for Parasitology serum bank (NRCP: Montreal, Canada). These sera had been screened using an 'in-house' *S. mansoni* and *S. haematobium* ELISA followed by species-specific testing (https://www.mcgill.ca/tropmed/nrcp). NRCP sera from an additional 19 subjects found to be negative schistosomiasis (7 males, 12 females; age range19:66 years) were used as controls (the limited demographic information available can be found in Table 1A & B).

Histology

Mouse livers collected at 6 or 12 weeks after infection were fixed in 10% buffered formalin and embedded in paraffin (Histopathology Core, Montreal General Hospital, Montreal, QC). Transverse sections (4 μm), were stained with hematoxylin-eosin and examined by light microscopy for histopathological changes and egg burden.

Initial Proteomic Analysis by SELDI

Individual murine and human serum samples were first analyzed by surface-enhanced, laser-desorption and ionization, time-of-flight mass spectrometry (SELDI TOF MS). In order to detect as many candidate biomarkers as possible, fractionated serum samples were applied to Immobilized Metal Affinity Capture (IMAC30), Cation Exchange (CM10), and reverse-phase hydrophobic H50 Protein Chip arrays (Bio-Rad Laboratories, Hercules, CA) as previously

described (Ndao et al., 2010). Briefly, ProteinChip serum fractionation kit (Bio-Rad) was used to define six pH fractions prior to MS analysis using 96-well Q-Ceramic HyperD F resin filtration plates. To decrease complexity, 20 µL of each sample were first incubated with 30 µL of U9 buffer (9 M urea 2% CHAPS, and 50 mM Tris-HCl, pH 9) for 20 minutes. Then, samples were diluted with 50 µL of U1 buffer [1 M urea, 2% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM Tris-HCl, pH 9] and added to pre-equilibrated filtration plates incubated on a MicroMix machine (MicroMix 5; Diagnostic Products Company, Los Angeles, CA) for 30 minutes. Five fractions were collected by serial elution in buffers of decreasing pH (pH9, 7, 5, 4, 3). The wash buffers used were #1 (50 mM Tris-HCl, 0.1% OGP, pH 9), #2 (50 mM HEPES, 0.1% OGP, pH 7), #3 (100 mM Na acetate, 0.1% OGP, pH 5), #4 (100 mM Na acetate, 0.1% OGP, pH 4) and #5 (50 mM Na citrate, 0.1% OGP, pH 3). A final elution by vacuum using an organic buffer yielded fraction #6 (33.3% isopropanol, 16.7% acetonitrile, and 0.1% trifluoroacetic acid). All fractions were stored at -80 ºC until analyzed.

Sample Preparation for LTQ-Orbitrap Velos

The LTQ-Orbitrap Velos analysis was performed in the Clinical Proteomics Platform, McGill University. Pooled serum samples (1 µL/lane) from control (12 weeks) as well as acutely (6 weeks) and chronically infected mice (12 weeks) were loaded onto precast 10% mini-protean TGX precast gels (BioRad, Mississauga, ON) and run for 45 min at 200 V. Gels were then stained and destained using standard Coomassie procedures (Chrambach et al., 1967). Each lane was sliced into 20 horizontal bands that were processed for proteomic analysis as previously described (Piersma et al., 2013). Briefly, bands were placed in 1.5 mL tubes and exposed to 40 µL of 100 mM NH_4HCO_3 for 5 minutes followed by centrifugation at 14,000 × g for 10 min.

After aspiration of the liquid, the gel pieces were vortexed with 40 µL of 100 mM NH_4HCO_3/50% acetonitrile (Sigma-Aldrich, St Louis, MO) for 10 minutes to shrink the gel. The gel pieces were dried in a microplate at 60°C in a vacuum concentrator for 30 minutes to remove excess acetonitrile and then placed in 40 µL of 10 mM dithiothreitol (DTT)/100 mM NH_4HCO_3 (Sigma-Aldrich) at 56°C in a closed water bath for 60 min. Gel pieces were then alkylated in 40 µL of 55 mM iodoacetamide + 100 mM NH_4HCO_3 for 45 min in 1.5 mL tubes in the dark at room temperature. After centrifugation at 14,000 x g for 15 min, the fluid was aspirated and gel pieces were washed in the same 1.5 mL tube in 100 µL of 100 mM NH_4HCO_3. Tubes were then centrifuged at 14,000 x g for 10 min, followed by removal of the fluid and washing of the gel in 40 µL of 100 mM NH_4HCO_3 for 5 min at room temperature after which 40 µL of acetonitrile (ACN) was added to make 1:1 solution and incubation was continued for 15 min at room temperature. This last wash-step with 100 µL of 25mM NH_4HCO_3 in 50% ACN was repeated once then the gel pieces were dried (60°C as above) and digested with 10-20 µL (enough to cover pieces) of trypsin (Roche, Mannheim, Germany) overnight at 37°C (12.5 ng/µL). After digestion, peptides were extracted with 100 µL 1% formic acid and vortexing at 37°C for 15 min. Tubes were then centrifuged at 14,000 x g for 15 min, and the supernatants were transferred to new 1.5 mL tubes (first extraction). A second extraction was performed with 100 µL 5% formic acid/50% acetonitrile, (Sigma-Aldrich) and, after centrifugation (14,000 x g for 15 minutes), the two extraction supernatants were pooled. The pooled supernatants were then dried in a vacuum centrifuge (Hermlelabortechnik, Germany) for ~1-2 hours at 50°C. Dried samples were re-suspended in 40 uL 0.05% formic acid and stored at −20°C until used for MS analysis.

Velos parameters

The samples were analyzed using a Thermo Easy-nano LC (Thermo Scientific, Bremen, Germany) connected to LTQ-Velos Orbitrap. Tryptic peptides were eluted at a flow rate of 300 nL/min: taking 60 min to go from 8% to 45% acetonitrile containing 0.1% (v/v) formic acid; 5 min at 80% and finally 15 min back to 0%. The total run time for each sample was 80 min. MS spectra were acquired with a resolution of 60,000 (scan range: 380-1400 m/z). The top 5-7 peptide signals (positive polarity) from each MS scan were submitted to MS/MS. The peptides were fragmented in the linear ion-trap by the collision-induced dissociation cell (1.0 m/z isolation width, 10 ms activation time, 35% normalized activation energy, Q value of 0.25) Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 15 s (Schenk et al., 2008; Olsen et al., 2005).

Protein identification

All MS/MS data were analyzed using Mascot (Matrix Science, London, UK; version 2.3.01) set up to search both schistosome and rodent protein databases (n_sch201202 and n_rodents20120120 respectively -All Entries) assuming that trypsin was used as the digestion enzyme. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 2.5 PPM. Carbamidomethyl of cysteine was specified as a fixed modification and oxidation of methionine was specified as a variable modification. After searching, Mascot .srf files were imported into Scaffold (Proteome Software Inc., Portland, OR: version 3.6.3) to validate the peptide/protein identifications. Identifications were accepted if the peptide achieved 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if the protein achieved 95.0% probability and contained at least one unique peptide. Protein probability were specified by the Protein Prophet algorithm (Nesvizhskii

et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS alone are not included in the displayed results.

Western blots

Mouse and human sera were separated by one-dimensional electrophoresis on 4-12% Bis-TrisNuPAGE gels (Invitrogen, Burlington, ON). Separated proteins were transferred to nitrocellulose membranes (Invitrogen, KiryatShmona, Israel) at 100 V for seven min, that were then stained with Ponceau S to verify transfer and as an initial loading control (Lanoix et al., 2012; Welinder et al., 2011;Aldridge et al., 2008; Collella et al., 2012). Membranes were then blocked with 5% skim milk in 0.05% Tween 20 in PBS (PBST) buffer for 1 h at room temperature (RT), followed by three washes with PBST for 5 min each. Membranes were incubated with a rabbit monoclonal antibody that recognizes human, mouse and rat carbonic anhydrase 1 (78% identity between mouse and human CA1) (Abcam) at a 1:150 dilution overnight at 4°C. Membranes were then washed three times with PBST for 5 min each. Incubations with HRP-conjugated anti-rabbit IgG were performed at 1:20,000 at RT for 1 h (Amersham Biosciences Co., Piscataway, NJ). Membranes were washed once in PBST for 15 minutes followed by 2 PBST washes of 5 minutes each then incubated in Super Signal West Pico detection solution for 1 min at RT (Pierce, Rockford, IL) and exposed to X-ray film. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal loading control. Membranes were stripped with 100 ml of stripping solution: 2 % sodium dodecyl sulfate (SDS), 62.5 mM tris HCL pH 6.7, 100 mM mercaptoethanol for 30 minutes at 55°C followed by re-probing with a mouse anti-GAPDH monoclonal (1:5000: Abcam). Blots were incubated with HRP-conjugated anti-mouse IgG at 1:10,000 at RT for 1 h (Amersham Biosciences Co.) and

exposed to X-ray film. Image J software (National Institutes of Health) was used to analyze densities of selected bands.

CA1 Enzyme-Linked Immunosorbent Assay (ELISA)

A human CA1 ELISA (Uscn, Life Science Inc. USA) was used according to the manufacturer's instructions. Briefly, 100 µl of standards or heat-inactivated serum samples (56°C for 30 minutes) were analyzed in duplicate wells and CA1 concentrations were calculated based on the internal standard curve. Samples that initially yielded results above the highest standard were diluted 2-fold with PBS and re-run. CA1 values are reported in mg/mL.

Statistical Analysis

The Statistical Package for the GraphPad Prism 6 (GraphPad Software, San Diego, California, USA) was used for statistical analysis. Data are expressed as means ± standard error of the mean (SEM). Unpaired t-tests were used to compare CA1 values between control and schistosomiasis patients. Statistical significance was defined as $P \leq 0.01$.

Results

Worm Burden and Histopathologic examination:

The number of worm pairs identified was similar in groups sacrificed at either 6 or 12 weeks post-infection (mean 15± 10 pair/mouse). Hematoxylin and eosin (H & E) stained liver sections at six weeks after the infection showed an early granulomatous response surrounding the entrapped eggs. Consistent with previous reports (Tom, et al., 2001), these granulomas appeared to consist primarily of macrophages and eosinophils surrounded by lymphocytes. At 12 weeks post-infection, liver granulomas were surrounded by large numbers of epithelioid cells and lymphocytes with occasional vacuolated giant cells. (Figure 1 A & B)

Strategy for Selecting the Most Promising Biomarkers

This study utilized two proteomic approaches to identify novel biomarkers for schistosomiasis. Since proteomic studies typically yield large numbers of candidate biomarkers, we moved back and forth between platforms and between murine and human samples to select candidates with the greatest potential to be clinically useful. Our first experiments used SELDI and pre-fractionated sera from the mice at 6 and 12 weeks post-infection to ask the simple question 'Are there differences in serum protein profiles following schistosomiasis infection?' This 'first look' revealed marked differences in the SELDI spectra between the uninfected and infected serum proteomes with at least 74 possible biomarkers based simply on m/z ratios. A representative example of discriminatory peaks are presented in Figure 2 were able to prove that the signal intensity of the peak increased with the prolonged time of infection.

Identification of Differential Proteins by LTQ-Velos Orbitrap

We next used the LTQ-Velos Orbitrap for brute-force MS analysis of 1-dimensional SDS-PAGE gel slices of pooled sera from uninfected and infected mice. This approach also generated large numbers of potential biomarkers (eg: > 1122 between uninfected and infected mice) but tentatively identified many of them based on MS microsequencing. This platform also permitted us to use Protein Identification Probability (PIP) algorithms based on percentage of total spectra, number of assigned spectra, number of unique spectra, peak intensities and percent coverage both to assess the level of confidence in the identifications and to generate semi-quantitative results. The latter capacity is based on the assumption that high-abundance proteins will produce more MS/MS spectra than low-abundance proteins (Bondarenko et al., 2002& Lu et al., 2007) so that comparing the number of spectra from the same protein between any two samples can serve as a crude relative quantitation of this protein (Wang et al., 2008).

Use of the Two Proteomic Platforms

Of the 1122 proteins tentatively identified by the Scaffold software, application of a 95% PIP filter brought the number down to 239. From these 239 differentially expressed proteins, serum levels of 22 were higher than control levels in both acute or chronic infection mouse serum and only eight were up- regulated in both the acute and chronic stages (Table 2A). Nineteen were higher in the control serum than either during acute or chronic infection with nine down-regulated in both acute and chronic stages (Table 2B). We then used the predicted molecular weights of the seventeen proteins that were consistently up- or down-regulated in schistosomiasis samples versus controls to query the mouse SELDI-TOF MS database for the corresponding peaks (± 5% mass range). Distinct SELDI peaks that differed substantially between infected and control sera were detected at m/z values of 28.85 (P value 0.07), 59.83 (P value 0.02), 145.32 (P value 0.01), kDa on the IMAC30 ProteinChips and at 23.17 (P value 0.02) kDa on the CM10 ProteinChips. Together, these data suggested that murine carbonic anhydrase 1 (mCA1: 28.85 kDa), proteasome subunit beta type-2 (59.83 kDa) and rat xanthine oxidoreductase mutant (145.32 kDa) were among the most promising biomarkers. The last SELDI peak of interest at 59.83 kDa was more complicated since this molecular weight corresponded to three proteins of similar molecular weight in the Orbitrap database: pancreatic alpha-amylase-like isoform 1, BC026782 protein and peptidoglycan recognition protein 2. When we used these tentative molecular weights to query our human serum SELDI database (data not shown), we found only one peak at 28.97 kDa on the H50 ProteinChip arrays that potentially corresponded to human homologue of carbonic anhydrase 1 (hCA1 28 kDa: Table 3).

Immunologic Confirmation by Western blot and ELISA

Western blot analysis of individual murine (Figure 3) and human serum samples (Figure 4) revealed striking reductions in serum CA1 levels in infected animals and patients compared to

uninfected control sera. In mice, when the timing of infection was known, there was a progressive decline in serum CA1 levels between 6 and 12 weeks after infection (Figure 3B). Serum concentrations of CA1 in human subjects with chronic schistosomiasis (n=17) were significantly lower than levels in normal controls (n=17) (74.2 ± 3.6 vs. 102.6 ± 9.9 pg/mL respectively; $p<0.011$) (Figure 5).

Discussion:

Schistosomiasis remains a serious health threat in many low- and middle-income countries. Despite the wide availability and relatively low cost of praziquantel, treatment is often delayed because the diagnostic tests available have low sensitivity both early and late in disease and because re-infection is common. There are currently no reliable biomarkers for active infection other than detection of living eggs in the stool or urine and egg-shedding can persist for years after treatment. There is an urgent need to develop new tests that can address some of these diagnostic 'gaps'. Long considered to be a research tool, mass spectrometry has recently entered the clinical microbiology laboratory for the rapid identification of pathogens (Fournier et al., 2013). In this work, we used two proteomic platforms and both murine and human serum samples to pursue novel biomarkers for schistosomiasis.

We used SELDI TOF MS to generate protein expression profiles from the serum of individual mice and patients (versus controls). Although these spectral data suggested the presence of major differences between infected and non-infected mice/patients, the translation of SELDI 'peaks' into identified candidate biomarkers can be very difficult due to the relatively low mass accuracy of this platform (Seibert et al., 2004). Not unexpectedly, there were also large differences between the SELDI spectra in infected mice and patients. To address these issues, we turned to the Orbitrap platform which provides higher mass accuracy and resolution in addition

to identification of the potential biomarkers. The Orbitrap data were then used to query both the murine and human SELDI spectral database for highly discriminatory biomarkers. This multi-step process yielded CA1 as a candidate 'negative' biomarker for both murine and human schistosomiasis that was subsequently confirmed by both Western blot and ELISA.

Carbonic anhydrases (CAs) are metalloenzymes that mediate the reverse hydration of CO_2 to H+ and HCO_3- and subsequent exchange of the H+ and HCO_3- ions for Na+ and Cl− across cell membranes (Swenson, 1991). These enzymes are present in all living things where they participate in a wide range of physiologic reactions including respiration, photosynthesis, pH regulation, electrolyte secretion and many biosynthetic reactions (Supuran, 2010). CAs can be grouped into five distinct genetic families or classes: α-, β-, γ-, δ- and ζ-. These families differ primarily in the metal ion used for catalysis; α-, β-, and δ-, CAs use zinc (Zn) ions at the active site, the γ-CAs may use iron (Fe) (Ferry et al., 2010), but they can also bind Zn or cobalt (Co) and the ζ-class uses cadmium (Cd) (Lane & Morel, 2000; Lane et al., 2005). α- CAs are the dominant form in humans and other animals (CA1 is one of α-CA isoforms). β-CAs are expressed in most species of bacteria, yeast, algae, plants, and some invertebrates such as nematodes and insects. γ-CAs have been reported in plants, archaea, and some bacteria (Elleuche & Poggeler 2010; Elleuche & Poggeler 2009). The last two CA classes: δ- and ζ-CAs are structurally similar to the β-CAs.

CAs have long been recognized as important drug targets and both activators and inhibitors are currently used as therapeutic agents for a wide range of conditions including infections, cancer, obesity, and glaucoma (Supuran, 2010; Güzel et al., 2009). Consistent with their central role in diverse biological processes, deficiencies in CA activity can lead to many different symptoms. In mice and humans, CA1 is found primarily in the red blood cells

(Konialis, et al., 1985), the colonic epithelium (Parkkila et al., 1994) and neutrophils (Campbell, 1 994). Abnormalities in CA1 isoenzymes are though to contribute to several types of anemia (Kuo et al 2005) and lower levels of CA1 mRNA and protein as well as total CA activity are found in the inflamed mucosa of patients with ulcerative colitis compared to controls despite the heavy infiltration of these tissues by neutrophils (Giovanni, et al., 1998; Robinson, et al., 1997). Deposition of *S. mansoni* eggs also causes acute and chronic inflammation of the colorectal mucosa (Mohamed et al., 1990). If reduction of CA1 expression and activity is a common feature of colonic inflammation, this could explain the reduction of CA1 levels we observed in the serum of *S. mansoni* infected mice and humans. Another possible explanation for the low serum CA1 concentration we observed in schistosomiasis is zinc deficiency. Serum zinc levels are low in subjects infected with *S. mansoni* and recover following treatment (El Hawy et al., 1993). Zinc is essential for the structure and activity of human carbonic anhydrases (Tripp et al., 2001). Although zinc levels were not measured in either the mouse or human sera used in this study, lower zinc levels *S. mansoni*-infected mice and patients may have contributed to the reduced levels of CA we observed.

In conclusion, serum CA1 appears to be a promising negative biomarker for human infection with *S. mansoni* at least during the chronic stage. Studies are planned to determine whether or not serum CA1 levels return to normal after praziquantel therapy and the kinetics of such normalization. We are curious to know if serum CA1 is reduced in subjects with other forms of schistosomiasis as well as other infectious and non-infectious conditions that cause bowel inflammation. It will also be important to determine the possible contribution of zinc deficiency to lower CA1 levels in schistosomiasis patients.

Acknowledgements

We are grateful to the Clinical Proteomics Platform, McGill University [http://www.clinprot.org/] for LTQ-Orbitrap Velos mass spectrometry analysis. We thank Nathalie Martel for providing technical assistance. Kardoush MI. is the recipient of a scholarship from the Government of Egypt.

Figure 1: Liver histology at six and 12 weeks after *S. mansoni* infection of C57Bl/6 mice with 200 cercariae by tail penetration (hematoxylin & eosin stain: 10x magnification) A). Hepatic granulomas at 6 weeks post-infection, the granuloma size is 24x42 µm. B). Hepatic granulomas 12 weeks post-infection, the granuloma size is 104.48x126.59 µm .The arrows indicate the *S. mansoni* granulomas.

Figure 1

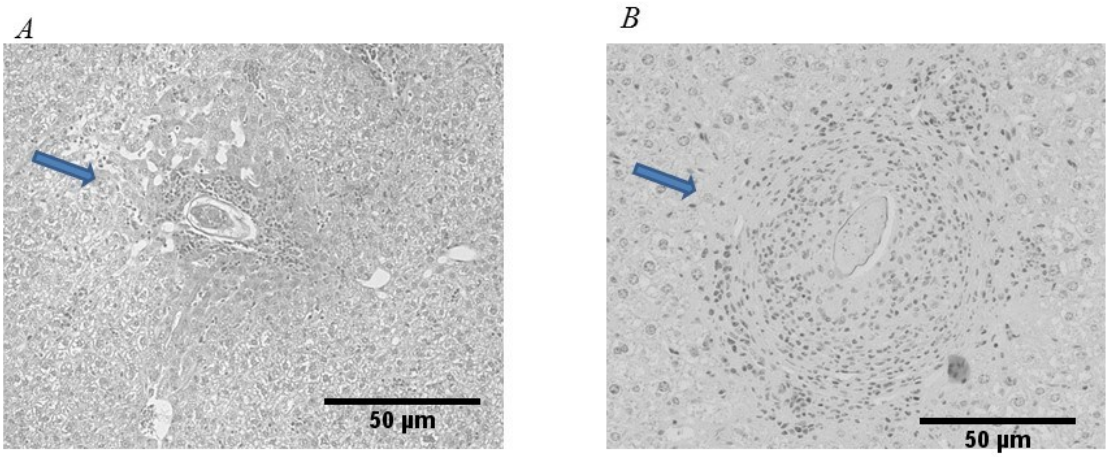

Figure 2: Example of a ~2794.38 Da candidate biomarker in infected mice that gradually increases from 6 to 12 weeks after infection (6W and 12W respectively; $p<0.01$) versus uninfected control animals (CTL). Serum SELDI-TOF MS mass spectra obtained for F6CSL (**F**raction **6**, **CM10** chip, **L**ow laser intensity) from infected mice (top 2 spectra) versus non-infected mice (bottom spectrum). The X-axis indicates the mass-to-charge ratio (m/z). The Y-axis: indicates the protein abundance expressed as absolute intensity (µA/laser pulse).

Figure 2

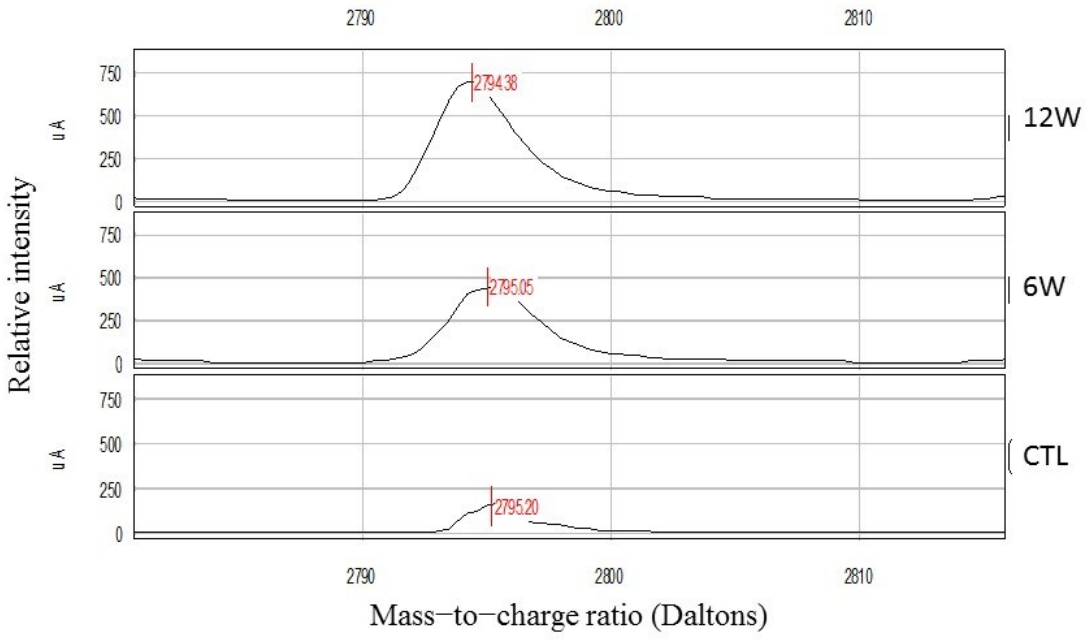

Figure 3: Immunologic validation CA1 as a candidate biomarker I A) Representative Western blot of CA1 in pooled sera from *S. mansoni*-infected mice (6 and 12 weeks post-infection: 6W and 12W respectively) and controls (C). B) Ponceau stained gel and C) Western blot of GAPDH served as a loading controls. D) Relative density of CA1 levels normalized to GAPDH.

Figure 3 A

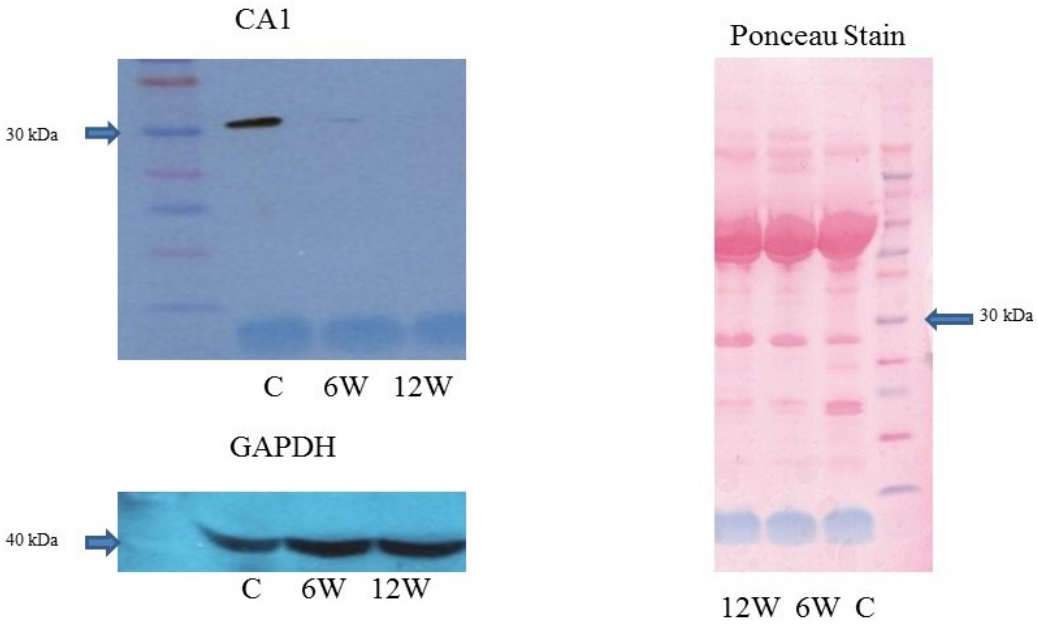

Figure 3 B

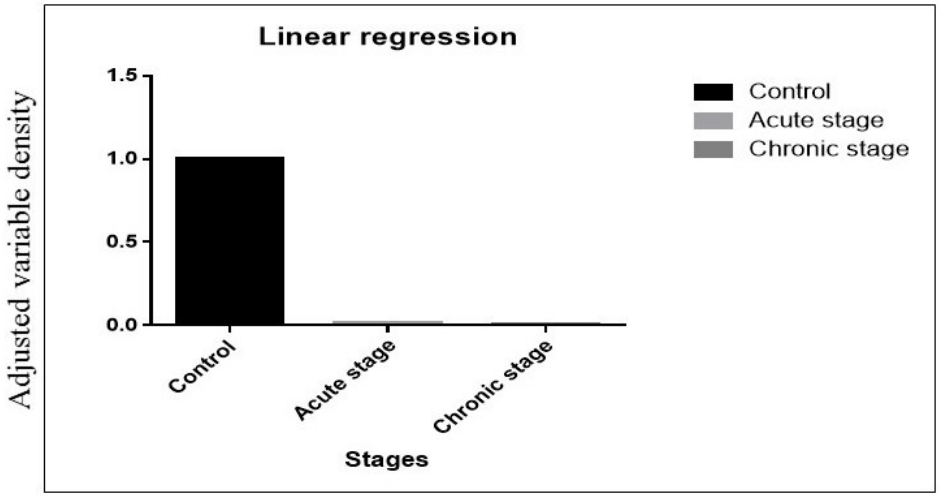

Figure 4: Validation of CA1 as a candidate biomarker in human serum A) Ponceau stained gel. B) Representative Western blot of CA1 in patients chronically-infected with *S. mansoni* (lanes I_1-I_8) versus controls (C_1-$_2$) and C) Western blot of GAPDH served as a loading controls D) Relative densities of CA1 bands in infected patients (I_1-I_8) versus controls (C_1-$_2$) ($p< 0.001$).

Figure 4A

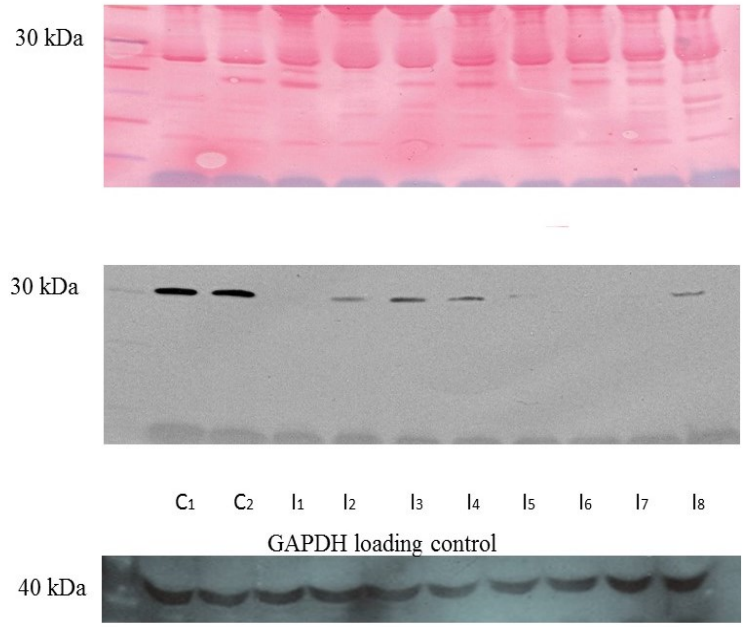

Figure 4B

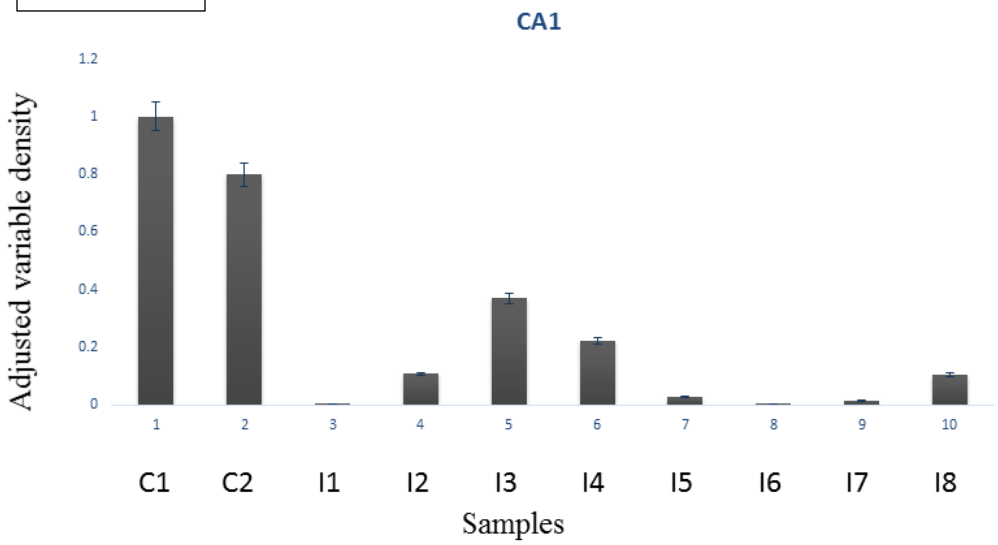

Figure 5: Serum CA1 levels in human patients chronically-infected with *S. mansoni* (74.2 ± 3.6 pg /ml) measured by ELISA were significantly decreased compared with healthy controls (102.6 ± 9.9 pg /ml, P=0.011; un-paired t-test).

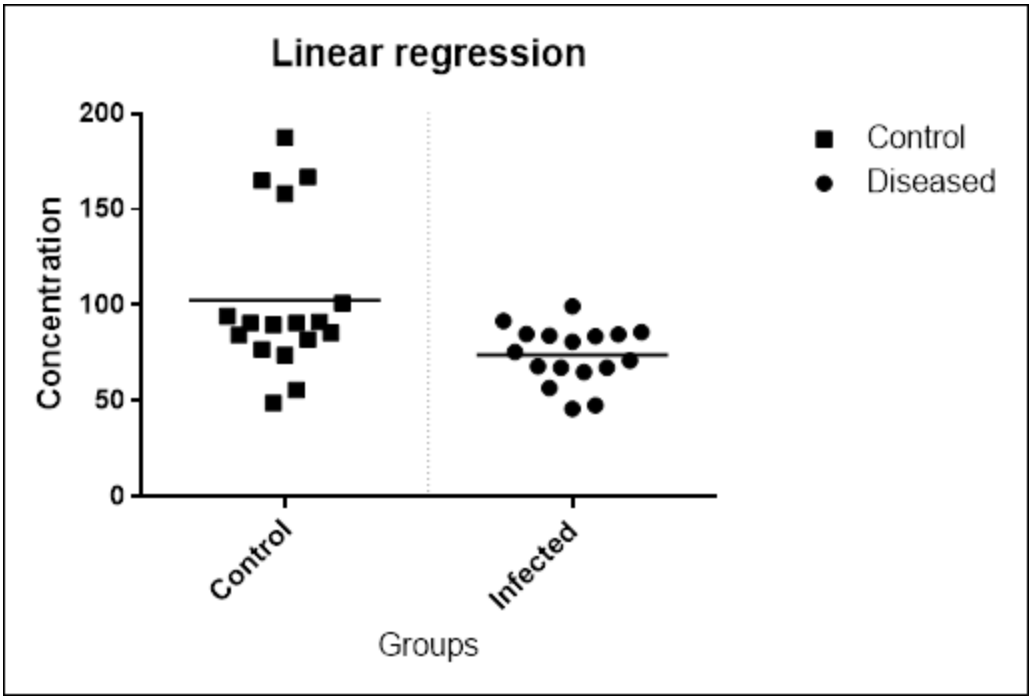

Table 1A: Demographic Information for Subjects with Schistosomiasis

Gender	Age	Schisto (OD)
M	18	0.51
M	22	1.06
F	42	1.22
M	10	1.6
F	18	1.31
M	41	0.69
M	19	0.72
F	26	0.72
M	60	0.59
F	50	0.81
M	52	0.62
M	17	1.04
F	22	0.96
F	25	1.65
M	16	1.17
M	41	0.72
M	18	1.01
F	20	1.62
M	47	1.14
M	10	0.88
M	22	1.17
M	21	1.1
F	48	1.31
M	23	0.9
F	15	1.22

• The cut-off values for Schisto ELISA: Negative: Optical Density (OD) < 0.35, Equivocal: 0.35 ≤ OD < 0.40, Positive: OD ≥ 0.40. The first 17 samples were used for ELISA and the last eight were used for Western blot.

Table 1B: Demographic Information for Control Subjects

Gender	Age	Schisto OD
F	39	0.14
M	61	0.08
F	21	0.08
M	34	0.07
F	44	0.08
F	33	0.1
F	66	0.07
F	64	0.1
F	25	0.17
F	59	0.12
M	51	0.14
M	20	0.18
F	21	0.16
M	35	0.1
M	55	0.1
M	25	0.08
F	19	0.08
F	40	0.11
F	59	0.09

The first 17 samples were used for ELISA and the last two were used for Western blot.

Table 2A: Eight proteins were increased in the sera of mice during both the acute and chronic stages of schistosomiasis compared to controls.

Proteins identified by LTQ-Orbitrap Velos	**Accession number**	**Molecular Weight**	**Taxonomy**
Myoglobin	21359820	17.07 kDa	Unknown
L Chain L, Crystal Structure Of The Complex Fab M75- Peptide	160285546	24.12 kDa	LK3 transgenic mice
Unnamed protein product	12843046	26.41 kDa	LK3 transgenic mice
Proteasome (prosome, macropain) subunit, beta type 1	37231712	26.40 kDa	unknown
Apolipoprotein F precursor	19527216	35.43 kDa	unknown
Leucine-rich alpha-2-glycoprotein precursor	16418335	37.43 kDa	unknown
Extracellular matrix protein 1 isoform 1 precursor	170295832	62.83 kDa	unknown
Maltase-glucoamylase, intestinal-like	354489684	403.52 kDa	unknown

Table 2B: Nine proteins were reduced in the serum of mice during both acute and chronic stages of schistosomiasis compared to controls.

Proteins identified by LTQ-Orbitrap Velos	**Accession number.**	**Molecular Weight**	**Taxonomy**
Proteasome subunit beta type-2	227116345	22.90 kDa	LK3 transgenic mice
Carbonic anhydrase 1	116063531	28.33 kDa	Unknown
Bisphosphoglycerate mutase	6680806	29.98 kDa	LK3 transgenic mice
Pancreatic alpha-amylase-like isoform 1	354486764	57.21 kDa	Unknown
BC026782 protein	20071242	58.07 kDa	LK3 transgenic mice
Peptidoglycan recognition protein 2	124245041	57.59 kDa	Unknown
Complement C2 precursor	157951694	84.74 kDa	Unknown
A Chain A, Crystal Structure Of Rat Xanthine Oxidoreductase Mutant	158428238	146.10 kDa	Buffalo rat
Murinoglobulin-2 precursor	50657404	161.59 kDa	Buffalo rat

Table 3: Predicted molecular weights of the seventeen serum proteins identified by Orbitrap that were consistently increased or decreased in murine schistosomiasis and SELDI-TOF MS peaks in both mouse and human serum that distinguished between infected and control samples.

Proteins identified by Orbitrap™	Accession number	Molecular Weight	Mouse SELDI	Human SELDI
Myoglobin	21359820	17.07 kDa	N/A	N/A
Proteasome subunit beta type-2	227116345	22.90 kDa	23.17	N/A
L Chain L, Crystal Structure Of The Complex Fab M75- Peptide	160285546	24.12 kDa	N/A	N/A
Unnamed protein product	12843046	26.41 kDa	N/A	N/A
Proteasome (prosome, macropain) subunit, beta type 1	37231712	26.40 kDa	N/A	N/A
Carbonic anhydrase 1	116063531	28.33 kDa	28.85	28.97
Bisphosphoglycerate mutase	6680806	29.98 kDa	N/A	N/A
Apolipoprotein F precursor	19527216	35.43 kDa	N/A	N/A
Leucine-rich alpha-2-glycoprotein precursor	16418335	37.43 kDa	N/A	N/A
Pancreatic alpha-amylase-like isoform 1	354486764	57.21 kDa	59.83	N/A
BC026782 protein	20071242	58.07 kDa	59.83	N/A
Peptidoglycan recognition protein 2	124245041	57.59 kDa	59.83	N/A
Extracellular matrix protein 1 isoform 1 precursor	170295832	62.83 kDa	N/A	N/A
Complement C2 precursor	157951694	84.74 kDa	N/A	N/A
A Chain A, Crystal Structure Of Rat Xanthine Oxidoreductase Mutant	158428238	146.10 kDa	145.32	N/A
Murinoglobulin-2 precursor	50657404	161.59 kDa	N/A	N/A
Maltase-glucoamylase, intestinal-like	354489684	403.52 kDa	N/A	N/A

References

Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ, 2008. The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. *Journal of Neuroscience Methods 172*(2):250-4.

Amiri P, Locksley RM, Parslow TG, Sadickt M, Rector E, Ritter D, McKerrow JH, 1992. Tumor necrosis alpha restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. Nature, 356, 604–607.

Bica I, Hamer DH, & Stadecker MJ, 2000. Hepatic schistosomiasis. *Infectious Disease Clinics of North America, 14*(3), 583-604.

Bondarenko PV, Chelius D, Shaler TA, 2002. Identification andrelative quantitation of protein mixtures by enzymatic digestion followedby capillary reversed-phase liquid chromatography-tandem mass spectrometry. *Analytical Chemistry, 74*(18), 4741–4749.

Byram JE, & von Lichtenberg F, 1977. Altered schistosome granuloma formation in nude mice. *The American Journal of Tropical Medicine and Hygiene, 26*(1), 944- 956.

Byram JE, Doenhoff MJ, Musallam R, Brink LH, & Von Lichtenberg F, 1979. Schistosoma mansoni infections in T-cell deprived mice, and the ameliorating effect of administering homologous chronic infection serum. II. Pathology. *The American Journal of Tropical Medicine and Hygiene, 28*(2), 274-285.

Campbell AR, Andress OL, & Swenson ER, 1 994. Identification and characterization of human neutrophil carbonic anhydrase. *J. Leukocyte Bioi, 55*(3):343-48.

Chrambach A, Reisfeld RA, Wyckoff M, & Zaccari J, 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Analytical Biochemistry, 20*(1), 150-154.

Doenhoff MJ, Chiodini PL, Hamilton JV, 2004. Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? *Trends in Parasitology, 20*(1), 35-39.

El Hawy AM, Abdel RM, Rozik S, Fawzy AF, el Gammal HA, 1993. Assessment of

serum copper and zinc levels in chronic Schistosoma mansoni patients with and without hepatic involvement before and after praziquantel therapy. *Journal of the Egyptian Society of Parasitology, 23*(1), 55-62.

El Ridi R, Wagih A, Salem R, Mahana N, El Demellawy M, Tallima H, 2006. Impact of interleukin-1 and interleukin-6 in murine primary schistosomiasis. *International Immunopharmacology, 6*(7):1100-8.

Elhag SM, Abdelkareem EA, Yousif AS, Frah EA, Mohamed AB, 2011. Detection of schistosomiasis antibodies in urine patients as a promising diagnostic maker. *Asian Pacific Journal of Tropical Medicine, 4*(10), 773-777.

Elleuche S, & Poggeler S, 2010. Carbonic anhydrases in fungi. *Microbiology, 156*(1), 23–29.

Elleuche S, Poggeler S, 2009. Evolution of carbonic anhydrases in fungi. *Curr Genet, 55*(2), 211–222.

Farah IO, Kariuki TM, King CL, Hau J, 2001. An overview of animal models in experimental schistosomiasis and refinements in the use of non-human primates. *Laboratory Animals, 35*(3):205-12.

Ferry JG, 2010. The γ class of carbonic anhydrases. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1804*(2):374-81.

Fournier PE, Drancourt M, Colson P, Rolain JM, La Scola B, Raoult D, 2013. Modern clinical microbiology: new challenges and solutions. *Nat Rev Microbiol., 11*(8):574-85.

Giovanni RF, Renzo L, Giuseppe C, Adriana F, & Sambuy MY, 1998. Carbonic anhydrase I reduction in colonic mucosa of patients with active ulcerative colitis. *Digestive Diseases and Sciences, 43*(9), 2086-2092.

Golledge CL, 1995. Schistosomiasis: beware delayed seroconversion. *The Medical journal*

of Australia, 163(8), 446-447.

Güzel O, Maresca A, Scozzafava A, Salman A, Balaban AT, Supuran CT, 2009. Discovery of low nanomolar and subnanomolar inhibitors of the mycobacterial β-carbonic anhydrases Rv1284 and Rv3273. *Journal of Medicinal Chemistry, 52*(13), 4063-4067.

Hipgrave DB, Leydon JA, Walker J, Biggs BA 1997. Schistosomiasis in Australian travellers to Africa. *The Medical journal of Australia, 166*(6), 294-297.

Jones ME, Mitchell RG, Leen CL, 1992. Long seronegative window in schistosoma infection. *The Lancet, 340*(8834), 1549-1550.

Jordan P, & Webbe G, 1969. Human schistosomiasis. *Human schistosomiasis*.

Keller A, Nesvizhskii AI, Kolker E, Aebersold R, 2002. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Analytical Chemistry, 74*(20), 5383-5392.

Kinkel HF, Dittrich S, Bäumer B, Weitzel T, 2012. Evaluation of eight serological tests for

diagnosis of imported schistosomiasis. *Clinical and Vaccine Immunology, 19*(6), 948-953.

Konialis CP, Barlow JH, Butterworth PH, 1985. Cloned cDNA for rabbit erythrocyte carbonic anhydrase I: A novel erythrocyte-specific probe to study development in erythroid tissues. *Proceedings of the National Academy of Sciences, 82*(3), 663-667.

Kuo WH, Yang SF, Hsieh YS, Tsai CS, Hwang WL, Chu SC, 2005. Differential expression of carbonic anhydrase isoenzymes in various types of anemia. *Clinica Chimica Acta, 351*(1), 79-86.

Lane TW, & Morel FM, 2000. A biological function for cadmium in marine diatoms. *Proceedings of the National Academy of Sciences, 97*(9):4627-31.

Lane TW, Saito MA, George GN, Pickering IJ, Prince RC, Morel FM, 2005. Biochemistry A cadmium enzyme from a marine diatom. *Nature 435*(7038): 42-42.

Lewis FA, Stirewalt MA, Souza CP, Gazzinelli G, 1986. Large-scale laboratory maintenance of Schistosoma mansoni, with observations on three schistosome/snail host combinations. *The Journal of Parasitology*, 813-829.

Lu P, Vogel C, Wang R, Yao X, Marcotte EM, 2007. Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nature Biotechnology, 25*(1), 117-124.

Mohamed AR, Al Karawi M, Yasawy MI, 1990. Schistosomal colonic disease. *Gut,*

31(4), 439-442.

Moreels TG, Joris G, Bogers JJ, De Winter BY, Vrolix G, Herman AG, Van Marck EA, Pelckmans PA, 2001. Effect of Schistosoma mansoni-induced granulomatous inflammation on murine gastrointestinal Motility. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 280*(5), G1030-G1042.

Ndao M, Spithill TW, Caffrey R, Li H, Podust VN, Perichon R, Santamaria C, Ache A, Duncan M, Powell MR, Ward BJ, 2010. Identification of novel diagnostic serum biomarkers for Chagas' disease in asymptomatic subjects by mass spectrometric profiling. *Journal of Clinical Microbiology, 48(*4), 1139-1149.

Nesvizhskii AI, Keller A, Kolker E, Aebersold R, 2003. A statistical model for identifying

proteins by tandem mass spectrometry. *Analytical Chemistry, 75*(17), 4646-4658.

Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, Makarov A, Lange O, Horning S, Mann M, 2005. Parts per million mass accuracy on an Orbitrap mass spectrometer via a lock mass injection into a C-trap. *Mol Cell Proteomics 4*, 2010–2021.

Parkkila S, Parkkila AK, Juvonen T, Rajaniemi H, 1994. Distribution of the carbonic anhydrase isoenzymes I, II, and VI in the human alimentary tract. *Gut, 35*(5), 646-650.

Pearce EJ, & MacDonald AS, 2002. The immunobiology of schistosomiasis. *Nature Reviews Immunology, 2*(7), 499-511.

Piersma SR, Warmoes MO, de Wit M, de Reus I, Knol JC, Jiménez CR, 2013. Whole gel processing procedure for GeLC-MS/MS based proteomics. *Proteome Science,11*(1):17.

Robinson CE, Kottapalli V, D'astice M, Fields JZ, Winship D, Keshavarzian A, 1997. Regulation of neutrophils in ulcerative colitis by colonic factors: a possible mechanism of neutrophil activation and tissue damage. *Journal of Laboratory and Clinical Medicine, 130*(6), 590-602.

Schenk S, Schoenhals GJ, de Souza G, Mann M, 2008. A high confidence, manually validated human blood plasma protein reference set. *BMC Medical Genomics, 1*(1), 41.

Seibert V, Wiesner A, Buschmann T, Meuer J, 2004. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI TOF-MS) and ProteinChip® technology in proteomics research. *Pathology-Research and Practice, 200*(2), 83-94.

Supuran CT, 2010. Carbonic anhydrase inhibition/activation: trip of a scientist around the world in the search of novel chemotypes and drug targets. *Current Pharmaceutical Design, 16*(29), 3233-3245.

Swenson ER, 1991. Distribution and functions of carbonic anhydrase in the gastrointestinal tract. In *The Carbonic Anhydrases* (pp. 265-287). Springer: US.

Tripp BC, Smith K & Ferry JG, 2001. Carbonic anhydrase: new insights for an ancient enzyme. *Journal of Biological Chemistry, 276*(52), 48615-48618.

Visser LG, Polderman AM, & Stuiver PC, 1995. Outbreak of schistosomiasis among travelers returning from Mali, West Africa. *Clinical Infectious Diseases, 20*(2), 280-285.

Wang M, You J, Bemis KG, Tegeler TJ, Brown DP, 2008. Label-free mass spectrometry-based protein quantification technologies in proteomic analysis. *Briefings in Functional Genomics and Proteomics, 7*(5), 329–339.

Welinder C, & Ekblad L, 2012. Coomassie staining as loading control in Western blot analysis. *J Proteome Res, 10*(3), 1416-1419.

Whitty CJ, Mabey DC, Armstrong M, Wright SG, Chiodini PL, 2000. Presentation and outcome of 1107 cases of schistosomiasis from Africa diagnosed in a non-endemic country. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 94*(5), 531-534.

CONNECTING STATEMENT II

In manuscript II, I described the carbonic anhydrase 1 as a novel biomarker from *S. mansoni* infected sera. CA1 is down-regulated in both the acute and chronic stages of *S. mansoni* infection in infected mice and chronic stage of *S. mansoni* infection in humans. In manuscript III, I studied the protein profiles in the serum of patients infected with *Schistosoma haematobium* vs. controls. I was able to identify candidate biomarker profile that is specific to *Schistosoma haematobium* infection using SELDI-TOF. We applied ESI MS to identify some of these biomarkers.

CHAPTER IV

Mass spectrometric approaches for the identification of candidate serum biomarkers for infection with *Schistosoma haematobium*

Kardoush MI,[1,2,3] Thiam A, [4], Faye B[4], Gaye O[4], Ndao M[5], Ward BJ [5].

Affiliations:

[1]Institute of Parasitology, McGill University, Montreal, Quebec, Canada

[2]National Reference Centre for Parasitology, Research Institute of the McGill University Health Centre, Montreal General Hospital, Montreal, Quebec, Canada

[3]Department of Parasitology, Faculty of medicine, Benha University, Benha, Qalubia, Egypt

[4]Laboratoire Régional de Biologie Médicale de Sor, BP 226, Saint-Louis du Sénégal.

[5]JD MacLean Tropical Diseases Centre, Research Institute of the McGill University Health Centre, Montreal General Hospital, Montreal, Quebec, Canada

*Corresponding Authour

Brian J Ward

Research Institute of the McGill University Health Center

Glen site – EM3-3248

1001 Décarie Blvd,

Montréal, QC, H4A 3J1

Telephone: 514-934-1934 x42810

FAX: 514-934-8224

Email: brian.ward@mcgill.ca

Other Authour emails: MI (manal.hassan@mail.mcgill.ca), MN (mndao@mcgill.ca)

ABSTRACT

Background: Schistosomiasis is a common parasitic disease for which new serum biomarkers are needed to improve diagnosis in both early and late stages. In this study, we sought to identify novel serum protein/peptide biomarkers in patients with chronic *Schistosoma haematobium* infection. **Methods**: We applied Surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS) and electrospray ionization (ESI) MS to serum samples from 30 Senegalese *S. haematobium* patients and 55 uninfected controls from the same region. **Results:** A total of 82 discriminating m/z peaks were detected by SELDI-TOF that differentiated chronic *S. haematobium* patients from controls ($P<0.01$). These biomarkers could be used in various combinations to generate diagnostic algorithms that achieved 100% sensitivity and specificity. One of the most useful SELDI biomarkers, a 28 kDa peak up-regulated in the infected subjects, was identified as apolipoprotein A-I (Apo A-I) and validated its identity by both Western blot and ELISA ($p<0.01$). **Conclusion:** Elevated Apo A-I may be a novel serum biomarker for the diagnosis of *S. haematobium*. Identification of the most important SELDI-flagged biomarker peaks may be useful not only for the development of novel diagnostic tests for *S. haematobium* but may also give new insights into host-parasite biology and therapeutic approaches.

Keywords: **Schistosomiasis; SELDI-TOF; ESI; Mass spectrometry; Biomarkers; Proteomic**

Word Counts: **Abstract: 201**

Text: 3128

3 Figures/ 1 Table

Introduction

Schistosomiasis is a neglected tropical disease that has a major public health impact in 70 endemic countries. More than 258 million people are thought to be infected, causing at least 200,000 deaths every year. As many as 600 million people are at risk for acquiring schistosomiasis (Bergquist, 2002; Rasoamanamihaja et al., 2016; Richter et al., 2016; Wang et al., 2009). The World Health Organization (WHO) estimates the number of disability-adjusted life years (DALY) lost due to schistosomiasis is ~1.5 million per year (Gryseels et al., 2006; Pullan et al., 2014), 90% of which are lost in sub-Saharan Africa (Lopez et al., 2006; Rasoamanamihaja et al., 2016). Currently, schistosomiasis control efforts aim to reduce morbidity through periodic treatment with praziquantel (PZQ) (WHO, 2012). However, PZQ does not prevent re-infection, has only moderate activity against immature worms and is losing efficacy due to emerging resistance (McManus & Loukas, 2008).

Current diagnostic tests for *S. haematobium* infected patients include detection of eggs in urine samples and detection of either egg antigens or antibodies in body fluids (e.g.: urine or serum) (Doehring et al., 1983). Immunodiagnostic methods can confirm the presence of infection but are not useful before antibody production (Sandoval et al., 2006) and cannot differentiate a past, cured infection from a current infection. Molecular diagnostic methods that target and amplify schistosome DNA from urine could possibly offer high sensitivity and specificity. However, these methods are expensive, require significant operator skills and laboratory infrastructure, which can limit their use in endemic fields (Rosser et al., 2015). Several studies have used monoclonal antibodies to detect and quantify a negatively charged anodic antigen (CAA) and a positively charged cathodic antigen (CCA) in serum and urine of infected individuals (Deelder et al., 1980; Stothard et al., 2006; Van Dam et al., 2004; Van Lieshout et al.,

2000). Despite having low sensitivity in low prevalence locations, these assays have been used as a substitute for parasitological examinations in endemic areas with moderate and high prevalence of schistosomiasis (Van Dam et al., 2004; Van Lieshout et al., 2000). The available CCA tests have better performance in detection of *S. mansoni* than *S. haematobium* (Gomes et al., 2014). Cystoscopy has been established as the gold standard test for detecting the late complications of schistosomiasis. However, this technique is invasive (Santos et al., 2015). A non-invasive diagnostic method and a new generation of biomarkers are necessary for better diagnosis of schistosomiasis.

In the present study, we used two mass spectrometric approaches in parallel to identify candidate biomarkers of late *S. haematobium* infection. Mass spectrometry platforms have been used for the detection of biomarkers in other parasitoses including both protozoan (Ndao et al., 2010, Papadopoulos et al., 2004), and helminth infection (Rioux et al., 2008). They also have been used to analyze the changes on the endothelial surface of the portal vein induced by *Schistosoma bovis* in infected mice (De la Torre-Escuderoa et al., 2012), and to study the metabolic fate of antiparasitic drug in the host and the parasite (Vokral et al.,2013).

The aim of this study is to assess whether protein profiles can distinguish between late *S. haematobium* infection and uninfected subjects from the same region, and target the most important proteins for identification, We followed a structured approach beginning with high throughput SELDI TOF MS analysis followed by ESI MS microsequencing and confirmatory immunologic testing (western blot, ELISA). This approach revealed serum Apo A-I as a powerful biomarker in chronically-infected patients.

Materials and methods

Samples

Samples were collected from the village of Ndombo, Medical District of Dagana, Saint-Louis, Senegal. The subjects were informed of the objectives of the investigation and signed informed consent to provide urine and 5 ml of blood. The filtration method was used to collect the eggs of *S. haematobium* from urine (WHO, 1991). Subjects were tested twice, 3 weeks apart before inclusion in the study. Infected patients received 40 mg/Kg of praziquantel for treatment after repeat samples were collected. The total number of samples was 85 including 55 negative controls, 30 *S. haematobium*-infected.

SELDI-TOF-MS

Serum samples were placed on ProteinChip™ arrays (Bio-Rad), fractionated and analyzed essentially as previously described (Rioux, 2008 & Ndao, 2009). Briefly, 96-well Q-Ceramic HyperD F resin filtration plates (Ciphergen Biosystems Inc., Fremont CA) were used to fractionate serum samples from each group by pH. Immobilized Metal Affinity Capture (IMAC30), Cation Exchange (CM10), and Reverse Phase (H50) Protein-Chip™ arrays were used to profile the fractionated samples as previously described (Ndao et al., 2010) according to the manufacturer's instructions (Bio-Rad). ProteinChip system (series 4000 Enterprise edition) was used to analyze the arrays (Bio-Rad). Each matrix and fraction combination was analyzed using optimal laser intensities. The intensity of the high laser was 3750 Da for all fractions. On the other hand, different intensities were used for the low laser. For example, 2500 Da for F1CS, 2400 Da for both F1IS & F6CS and 2300 Da for F6IS. The spectra were collected and analyzed separately. Each data set was calibrated using an equation generated from the spectra of protein standards which was collected at the same laser intensity used in sample data collection. The external calibration standards were: insulin (bovine) (5,733.58 Da), ubiquitin (bovine) (8,564.8 Da), cytochrome C (bovine) (12,230.9 Da), superoxide dismutase (bovine) (15,591.4Da) and

myglobin (equine cardiac) (16,951.5 Da). High: peroxidase (horse radish) (43,240Da) and albumin (bovine serum) (66,433Da). The baseline for all data was set at 15 times expected peak width and the noise was set at 2,000 Da for low laser energy or 10,000 Da for high laser energy. Sample spectra for each group were normalized using total ion current between 2000-100,000 Da for low laser intensity, and 10,000-200,000 Da for high laser intensity. An external normalization coefficient of 0.2 was applied for both conditions. Any spectra that did not fall within twice the overall average normalization factor was excluded from the analysis. Ciphergen Express™ software version 3.0.6 was used to analyze the spectra in two steps. First, the Cluster Wizard™ tool was used to align the peaks of spectra from different samples (expression difference mapping). In the first pass analysis, automated detection identified peaks with a single to noise (S/N) ratio of ≥ 5. The Mann-Whitney test was used to identify statistically significant differences between control and infected samples. In the second step the peaks with statistically significant differences with *p*-values ≤ 0.05 were inspected and relabeled manually. During the second pass analysis, peaks with a S/N ratio of ≥ 2 were retained. Potential biomarkers were defined when the peaks had *p*-values of ≤ 0.05 and Receiver Operation Characteristic (ROC) values of < 0.3 and > 0.7.

Zoom Fractionation

In order to decrease sample complexity for ESI analysis, isoelectric focusing was used as previously described, (ZOOM® Fractionator: Invitrogen, Carlsbad, CA) (Zuo et al., 2002). Fractionation allows the loading of larger amounts of protein leading to increased dynamic range, higher resolution and facilitating the identification of low abundance proteins (Zuo et al., 2001; Zuo & Speicher, 2002). Briefly, 650 µL of sample was loaded onto ZOOM® discs using the five different pH chambers (pH 3.0−4.6, pH 4.6−5.4, pH 5.4−6.2, pH 6.2−7.0 and pH

7.0−10.0) according to the manufacturer's instructions. The fractionated samples were concentrated/desalted by methanol-chloroform precipitation. Fractions were recovered in separate Eppendorf tubes, store at −80 °C untill use.

SDS-PAGE and in-gel digestion

The fractionated pooled serum samples (1 µL/lane) from control and *S. haematobium* infected patients were loaded onto 4-12% Bis-TrisNuPAGE gels (Invitrogen Life Technologies, Carlsbad, CA), and run for 45 min at 200 V. All gels were stained with colloidal Coomassie blue G-250 stain (colloidal blue staining kit; Invitrogen). Stained gels are digitalized and then comparing the intensities of stained spots to compare the amount of specific proteins visually. After that the protein spots were excised from the SDS gel. The gel bands were kept in 2% acetic acid until further analysis by mass spectrometry

Protein identification by mass spectrometry

The gel pieces were dried under the vacuum and then rehydrated in Trypsin Digest Solution (proteomics grade, Roche) at 37°C overnight. Peptides were extracted with a an extraction solution of 5 µl of acetonitrile and 0.1 % trifluoro acetic acid solution (50:50 v/v), after desalting with Millipore's C18 Zip Tips, the samples were resuspend in 10 µL 0.1% TFA and subjected to MS analysis. This was done using a soft ionization technique which allows mass spectrometric analysis of biopolymers such as proteins and peptides which are thermally unstable, polar, and non-volatile. Electrospray ionization (ESI) technique was used for this proteomic (Fenn et al., 1989).

ESI-TRAP:

Electrospray ionization (ESI; Proteomics Core Facility Bellini Life Science Center, McGill University) was used for MS analysis of selected bands from *S. haematobium* and

control. The sample proteins are dissolved in easily evaporated solvent, then injected in charged a capillary tube which is maintained at a high voltage (2-6kV), applied at the end of the tube. Passage of the analyte from this tube under the strong electric field results in an electrically-charged spray, with application of a nebuliser gas such as nitrogen, the spray passes from the capillary tip to the analyser region of the mass spectrometer. At the end the electric field strength in the charged droplets causes transfer of the ions at the surface of the droplets into the gaseous phase. The released ions are accelerated into the mass analyser for identification of the molecular mass and the intensity (Bruins, 1998). The resulting spectra were submitted to the database mining tool Mascot (http://www.matrixscience.com/) and searched against Homo sapiens (human) NCBInr 20,100,811 (11,613,246 sequences; 3,967,887,859 residues). The following search parameters were used: trypsin is the enzyme of digestion, one missed cleavage was allowed; carbamidomethylation as fixed modification and oxidation as variable modification, with unrestricted protein masses were considered along with peptide mass tolerance at ± 1.3 Da and fragment mass tolerances at ± 0.4 Da for an ESI-TRAP instrument. Protein identifications resulting from MASCOT ions scores had to be inside the 95% confidence level. As an internal control, one band (64 kDa) was taken from the ladder and identified as glutamate dehydrogenase.

Western Blots

Neat sera from *S. haematobium* infected patients and controls were separated by one-dimensional electrophoresis on 4-12% Bis-Tris NuPAGE gels (Invitrogen). Isolated proteins were trans-blotted onto nitrocellulose membrane. The proteins were transferred at 100 V for 1 h and blocked by 5% skim milk in 0.05% Tween 20 in PBS (PBST) buffer for 1 hour at room temperature, followed by three washes with PBST for 5 min each. Membrane was incubated with rabbit Apo A-I Antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution for

either 1 hour at room temperature or overnight at 4°C. The membrane was washed three times with PBST for 5 min each. Incubation with HRP-conjugated anti-rabbit IgG were done at a 1:100,000 dilutions at room temperature for 1 hour (Amersham Biosciences Co., Piscataway, NJ). The membrane was washed one time in PBST for 15 minutes followed by 2 washes for 5 minutes each then incubated in Super Signal West Pico detection solution (Pierce, Rockford, IL) and exposed to X-ray film. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal loading control. Membranes were stripped with 100 ml of stripping solution: 2 % sodium dodecyl sulfate (SDS), 62.5 mM tris HCL pH 6.7, 100 mM mercaptoethanol for 30 minutes at 55°C followed by re-probing with a mouse anti-GAPDH monoclonal (1:5000: Abcam). Blots were incubated with HRP-conjugated anti-mouse IgG at 1:10,000 at RT for 1 h (Amersham Biosciences Co.) and exposed to X-ray film. Image J software (National Institutes of Health) was used to analyze densities of selected bands.

Apo A-I Enzyme-Linked Immunosorbent Assay (ELISA)

A human ApoA-1 ELISA (Mabtech, Inc. USA) was used according to the manufacturer's instructions. Briefly, 100 µl of standards or heat-inactivated serum samples (56°C for 30 minutes) were analyzed in duplicate wells and ApoA-1 concentrations were calculated based on the internal standard curve. ApoA-1 values are reported in ng/mL.

Statistical Analysis

The Statistical Package for the GraphPad Prism 6 (GraphPad Software, San Diego, California, USA) was used for statistical analysis. Data are expressed as means ± standard error of the mean (SEM). Unpaired t-tests were used to compare ApoA-1 values between control and schistosomiasis patients. Statistical significance was defined as $P \leq 0.01$.

Results

Serum protein profiles and data processing

Using pre-established criteria (p-value ≤ 0.05), ROC value <0.3 and >0.7), the SELDI analysis generated a total of 82 candidate biomarkers across the 6 fractions tested that were useful to distinguish between control vs. *S. haematobium* subjects (Table 1). Of these, 82 protein peaks, 15 peaks were up-regulated and 67 were downregulated. A representative sample of discriminatory peaks is presented in Figure 1. The differential expression of one peak in particular (28970 m/z) was highly significant between control and *S. haematobium* (p-value = 0.0007 and ROC value = 0.78) (Figure 2a). This peak was prioritized for protein identification.

Zoom fractionation SDS PAGE Gel and Protein Identification by ESI

After fractionation of the gel band containing the discriminatory 28 kDa protein and ESI microsequencing, a Mascot search revealed the proposed protein to explain the SELDI peak at 28 kDa in the *S. haematobium* infected sera as ApoA1.

Validation of Apo A-I biomarkers by Western Blot and ELISA

Western blot analysis using antisera to human Apo A-I detected a band at ~28 kDa; the intensity of this band *S. haematobium* infected patients sera was four-times the same band in control sera (Figure. 2b). Serum concentrations of Apo A-I in human subjects with chronic schistosomiasis (n=15) were significantly higher than levels in uninfected subjects (n=14) (6.81± 1.62 vs. 5.06 ± 1.23 ng/mL respectively; p<0.01) (Figure 3).

Discussion

This study proved that the sera of patients infected with *S. haematobium* have characteristic protein profile that is different from healthy controls. This different protein profile could be used for the diagnosis of schistosomiasis. Diagnosis of *S. haematobium* depends on the microscopic visualisation of the eggs in urine (Doehring et al., 1983). Immunological methods

such as ELISA are also used to detect host antibodies. However, these methods are limited in their sensitivity and specificity (Sandoval et al., 2006). To identify diagnostic and therapeutic biomarkers in many diseases, the best place to start with is serum proteome. SELDI-TOF MS is a proteomic technology for biomarker discovery that is used for rapid and sensitive analysis of protein mixtures (Issaq et al., 2002; Scebba et al., 2016). This study used SELDI-TOF MS to search for new biomarkers in the serum of *S. haematobium* infected patients. We have found that there are many biomarkers that may potentially be used to differentiate the infected from the control groups.

The presence of abundant proteins such as immunoglobulins and albumin often complicates the detection of the less abundant proteins. In order to overcome this problem and allow the detection of less abundant proteins, we employed fractionation to separate the proteins according to pH. Sequencing with ESI followed by a Mascot search revealed ApoA-1 protein from the spot taken from *S. haematobium* infected sera.

ApoA-1 (28 kDa), is a major protein component of high-density lipoprotein (HDL) in plasma. The main functions of ApoA-I include interaction with cellular receptors, promote cholesterol efflux from tissues to liver for excretion and activation of lecithin/cholesterol acyltransferase (Pirillo et al., 2015). Furthermore, it has an anti-clotting effect because it was isolated as a prostacyclin (PGI2) stabilizing factor (Yui et al., 1988). Roheim (1986) reported that the main role of ApoA-1 is the formation of HDL. The study also reported that low levels of this protein are a predisposition factor for development and progression of coronary heart disease. The liver and small intestine are main sites for ApoA-1 synthesis and secretion (Pirillo et al., 2015). Several studies have shown that human schistosomiasis modifies plasma lipid composition (Dimenstein et al., 1991) and metabolism (Da Fonseca et al., 2014; Silva et al.,

2002). Sprong et al. (2006) showed that even though schistosomes need cholesterol for their growth and egg production, they do not synthesize it themselves. Schistosomes have metabolic pathways defects, they do not synthesize fatty acids *de novo* and they use host serum lipoproteins to overcome this or to disguise themselves from the immune system of their hosts. The tegument of the parasite actively binds the host's low density lipoproteins. This provides the parasite with cholesterol and other lipids and might also help it evade the immune system (Bica et al., 2000; Brindley, 2005; Braschi et al., 2006). Ridi et al. (2004) and Tallima & Ridi (2005) reported that the main role of cholesterol in biomembranes is the sequestration of schistosomula surface membrane antigens. Doenhoff et al. (2002) indicated that infection with schistosomes reduced the total cholesterol level in the blood and modulated the host lipid metabolism in *S. mansoni* infected mice.

In the case of an impaired renal function, HDL-cholesterol and ApoA-1 in plasma are significantly changed (Graversen et al., 2008; Kaysen, 2009). The main pathological process in chronic *S. haematobium* infection occurs when schistosome eggs become trapped in the bladder wall and ureters causing chronic inflammation, which may in turn obstruct the ureters and damage the kidneys (Barsoum et al., 2013). According to Soto-Miranda and colleagues (2012), the kidney effortlessly removes small HDL particles, while large HDL remain in the plasma of patients with proteinuria. Proteinuria is indirect disease marker that is commonly used to identify individuals at risk of *S. haematobium* infection in endemic settings (Houmsou et al., 2013). Renal failure causes an increase in the ApoA-1 (Duval et al., 1989). Subramanian and colleagues (1999) have shown an association between *S. haematobium* and urinary tract diseases such as hydronephrosis. Given these previous findings from different studies, we postulate that an

increase levels of Apo-A1 could be as a result of urinary tract complications that is associated with schistosomiasis.

In our previous study, we have identified CA1 using Orbitrap as a negative serum biomarker for the diagnosis of *S. mansoni* infection. CA1 has similar molecular weight as ApoA1, and both are about 28kDa. In the current study, CA1 was not detected in the serum of *S. haematobium* infected patients neither by SELDI or ESI. This is could mean that CA1 is a biomarker for *S. mansoni* can also be used to differentiate between *S. mansoni* and *S. haematobium*.

In conclusion, novel biomarker candidate proteins differentially expressed in patients infected with *S. haematobium* compared to control serum samples were identified using two proteomic approaches, SELDI-TOF and ESI. Serum ApoA-1 appears to be a promising biomarker for human infection with *S. haematobium* during the chronic stage. Studies are planned to determine whether or not serum ApoA-1 levels return to normal after praziquantel therapy. We are curious to know if serum ApoA-1 is elevated in subjects with other forms of schistosomiasis as well as other parasitic diseases.

Additional analysis of a larger set of individual samples is required to further confirm the high level of the identified serum proteins.

FIGURES AND TABLES

Figure 1: Example of a ~6641 Da candidate biomarker in serum samples of *S. haematobium* infected patients ($p<0.01$) versus uninfected controls (CTL). Serum SELDI-TOF MS mass spectra obtained for F6CSL (**F**raction **1**, **CM10** chip, **L**ow laser intensity) from infected (top 2 spectra) versus non-infected (bottom spectrum). The X-axis indicates the mass-to-charge ratio (m/z). The Y- axis: indicates the protein abundance expressed as absolute intensity (µA/laser pulse).

Fig. 1

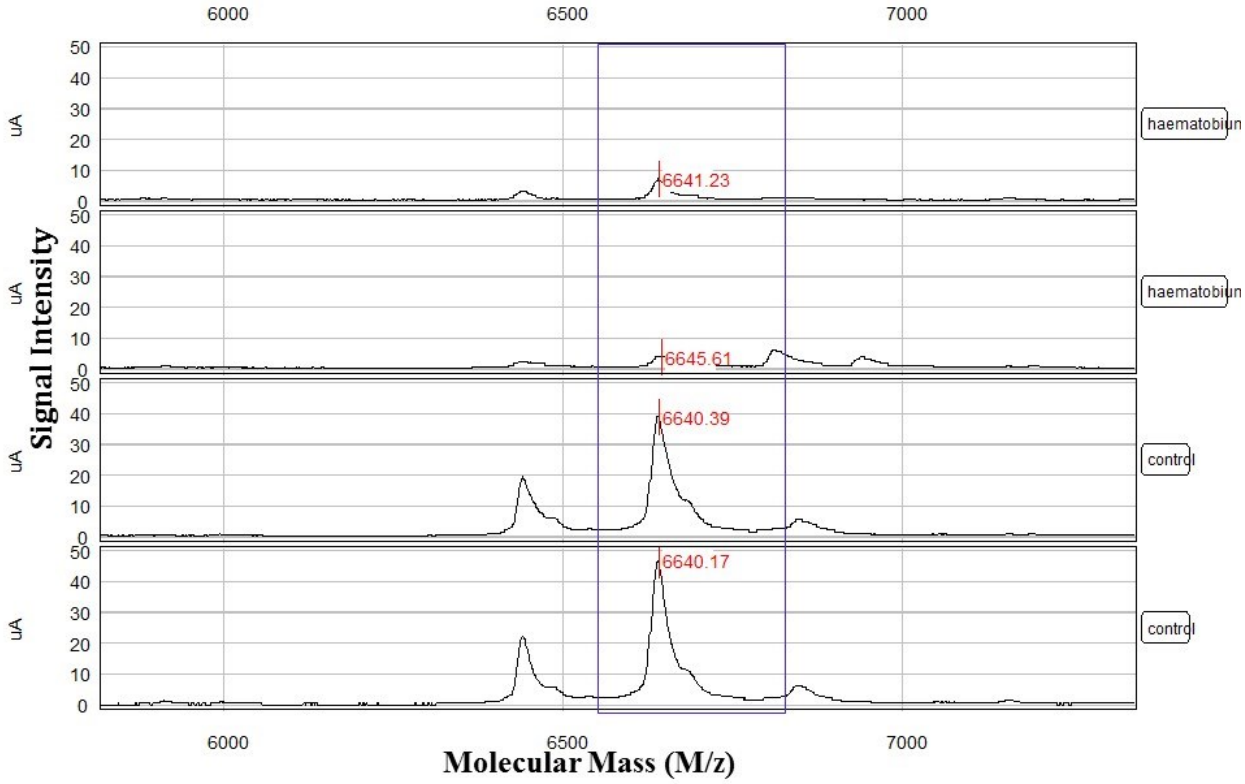

Figure 2: Immunologic validation ApoA-1 as a candidate biomarker I A) ~28 kDa candidate biomarker in serum samples of *S. haematobium* infected patients versus uninfected controls (CTL) B). Representative Western blot of ApoA-1 in pooled sera from *S. haematobium*-infected samples and controls. C) Western blot of GAPDH served as a loading controls. D) Relative density of ApoA-1 levels normalized to GAPDH.

Fig. 2

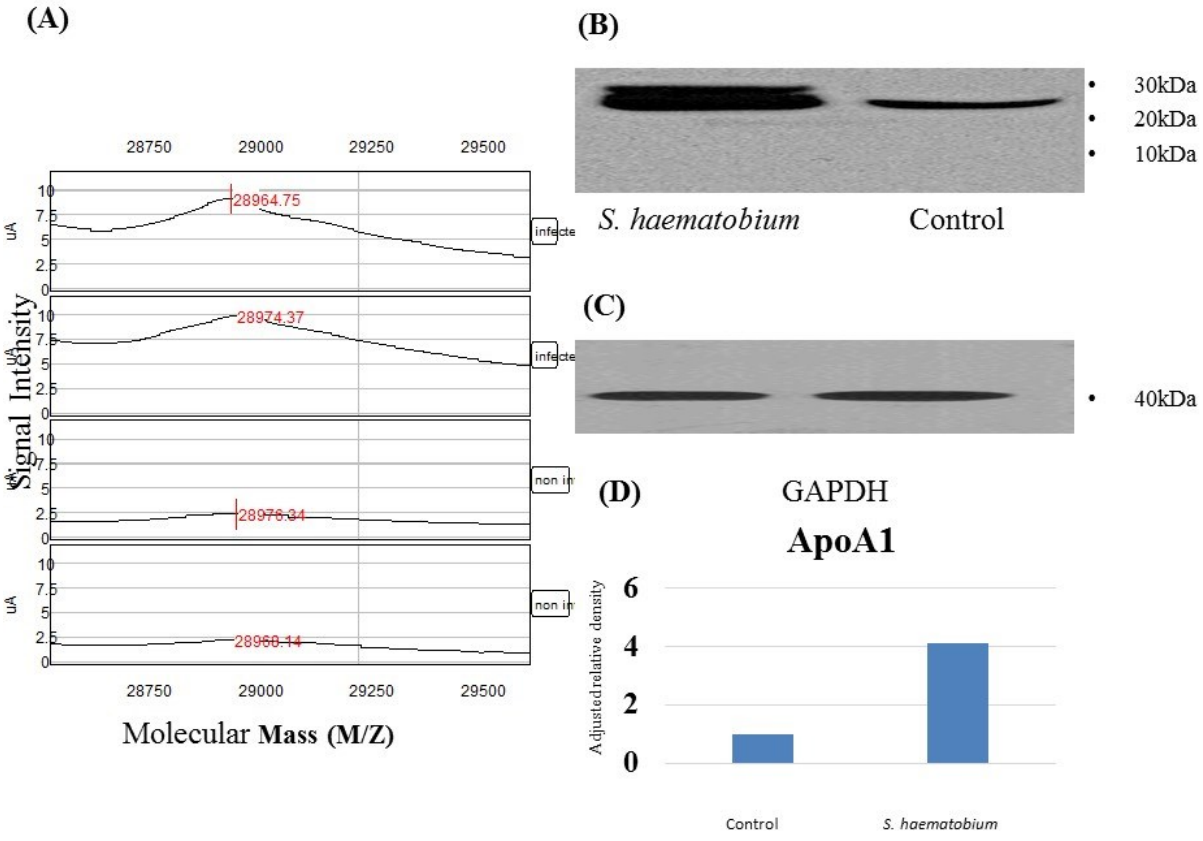

Figure 3: ELISA was used to measure serum level of ApoA-1. Serum ApoA-1 levels in patients with infected with *S. haematobium* were significantly increased when compared with the healthy controls (6.81± 1.62 vs. 5.06 ± 1.23 ng/mL respectively; $p<0.01$; un-paired t-test). Results of all 29 analyzed serum samples: boxplot of the ApoA-1 levels in *S. haematobium* infected patients (black bars) compared to controls (gray bars). Median values are indicated by horizontal lines within the boxes. The lower part of the box represents the first quartile, and the upper part represents the third quartile. The vertical width of the central box symbolizes the inter-quartile deviation. The protruding vertical lines from the box represents the minimum and the maximum values of the data.

Fig. 3

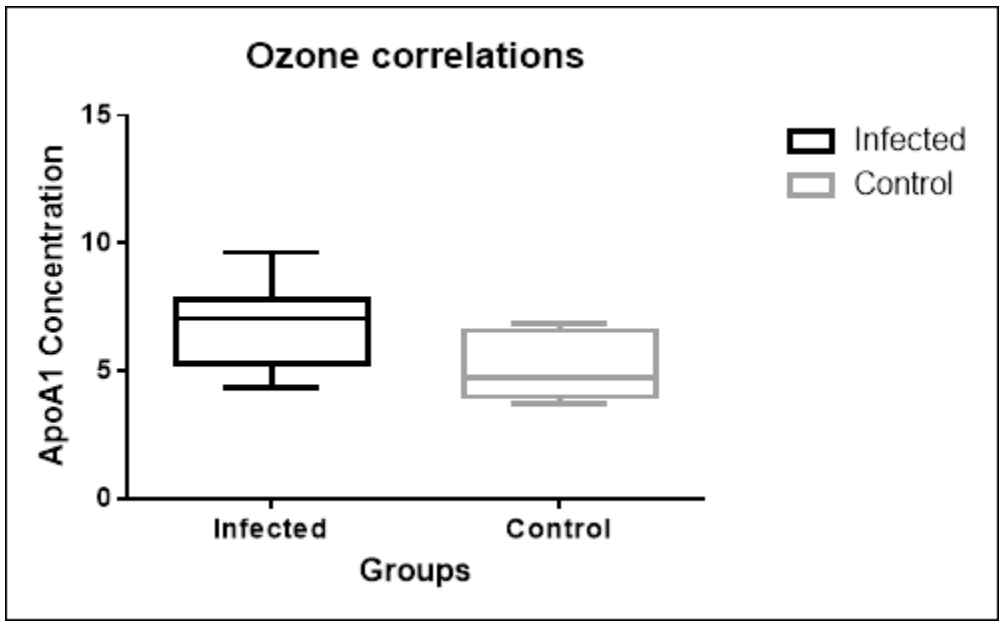

Table 1: Mass (m/z), p values, and ROCs for detected differentially expressed peptides/proteins between *S. haematobium* infected patients and healthy controls (* the highlighted peaks are upregulated in the disease).

Fraction	Index	p value	ROC	M/Z Average
F1CSH	3	0.003681	0.77	15107.91
	4	0.011385	0.733077	15180.31
	5	0.010846	0.714615	15224.36
	6	0.007664	0.751538	15320.33
	11	0.015876	0.285385	89729.66
	13	0.006248	0.278462	193586.7
F1CSL	3	0.024031	0.310047	3980.375*
	11	0.003204	0.782692	5913.488*
	21	0.007548	0.735479	1524.422*
	19	0.038789	0.697802	7943.158*
	26	0.011951	0.716641	6642.186
F1ISH	2	0.003921	0.778986	10784.84*
	4	0.016489	0.753623	11328.29
	5	0.016489	0.778986	11489.01
	6	0.012343	0.753623	11692.35
	7	0.028568	0.753623	12649.22
	9	0.031189	0.702899	13052.38
	10	0.040326	0.728261	13784.73*
	11	0.01812	0.246377	14911.65*
	12	0.014988	0.246377	15130.54
	13	0.00487	0.75947	15257.09
F1ISL	15	0.005637	0.242908	7532.141
	19	0.004462	0.223404	12910.15
	20	0.007927	0.718085	15176.4*
	21	0.00838	0.737589	15310.81*
	22	0.015936	0.698582	15919.96*
	23	0.015132	0.737589	16086.05
F1HSH	8	0.034292	0.32197	13318.56
	1	0.02274	0.281281	11584.2*
	7	0.039807	0.671196	67304.8*
F1HSL	8	0.041056	0.297276	4661.04
F6CSH	7	0.017921	0.727083	15109.74

	8	0.037613	0.704167	15315.39
	9	0.02114	0.7125	15848.84
	10	0.02485	0.7125	15976.07
	11	0.029111	0.7125	16004.28
	12	0.033987	0.7125	16046.95
	13	0.02114	0.735417	16843.14
	14	0.041562	0.285417	17372.51*
	15	0.03229	0.308333	17576.44
	23	0.017921	0.727083	24683.68
	25	0.012028	0.239583	33407.4
	26	0.008418	0.239583	34596.27
	27	0.030665	0.33125	36867.48
	29	0.010695	0.2625	44086.78*
	31	0.027626	0.285417	59245.04
	32	0.020015	0.2625	59700.91
	33	0.029111	0.285417	60412.25
	34	0.02114	0.2625	62012.03
	35	0.045856	0.354167	66936.47
	36	0.037613	0.322917	73624.43
	37	0.006584	0.23125	79391.97
	39	0.033987	0.285417	101427.4
	41	0.029111	0.285417	118010.8
	42	0.012748	0.285417	134710.8
	44	0.012748	0.285417	186338.5
F6CSL	6	0.008618	0.255102	3886.982
	13	0.010759	0.277365	4464.722
	14	0.010184	0.232839	4534.927
	15	0.048008	0.321892	4622.539
	30	0.011363	0.255102	17406.48
F6ISH	1	0.025643	0.265306	10296.72
	4	0.044024	0.306122	11589.23
	5	0.004651	0.244898	11772.42
	24	0.022254	0.306122	116676.3
F6ISL	8	0.019591	0.26	6174.784
	9	0.021536	0.28	6189.505
	15	0.004361	0.24	11731.71
	16	0.001341	0.24	11902.33
	23	0.044076	0.36	21734.43

F6HSH	7	0.00073	0.784178	28970.9*
F6HSL	1	0.046142	0.334087	2574.242
	3	0.034781	0.334087	2857.765
	4	0.047996	0.346154	2972.197
	5	0.036241	0.328054	3022.799
	7	0.028211	0.309955	3554.957
	12	0.019052	0.297888	5422.927
	16	0.015897	0.708145	8457.431
	25	0.012606	0.279789	10733
	26	0.003593	0.22549	11549.17
	27	0.034781	0.315988	11704.15
	30	0.046142	0.334087	14802.14
	3	0.03478	0.33409	2857.76466
	4	0.048	0.34615	2972.19669
	5	0.03624	0.32805	3022.79904
	7	0.02821	0.30995	3554.9566
	12	0.01905	0.29789	5422.92681
	16	0.0159	0.70814	8457.431
	25	0.01261	0.27979	10733.0025
	26	0.00359	0.22549	11549.1689
	27	0.03478	0.31599	11704.1536
	30	0.04614	0.33409	14802.1399

References

Barsoum, R. S., Esmat, G., & El-Baz, T. (2013). Human schistosomiasis: clinical perspective: review. *Journal of Advanced Research, 4*(5), 433-444.

Bergquist, R., Al-Sherbiny, M., Barakat, R., & Olds, R. (2002). Blueprint for schistosomiasis vaccine development. *Acta Tropica, 82*(2), 183-192.

Bica, I., Hamer, D. H., & Stadecker, M. J. (2000). Hepatic schistosomiasis. *Infectious Disease Clinics of North America, 14*(3), 583-604.

Braschi, S., Borges, W. C., & Wilson, R. A. (2006). Proteomic analysis of the shistosome tegument and its surface membranes. *Memorias do Instituto Oswaldo Cruz, 101,* 205-212.

Brindley, P. J. (2005). The molecular biology of schistosomes. *Trends in Parasitology, 21*(11), 533-536.

Bruins, A. P. (1998). Mechanistic aspects of electrospray ionization. *Journal of Chromatography A, 794*(1), 345-357.

Da Fonseca, C. S. M., Pimenta Filho, A. A., dos Santos, B. S., da Silva, C. A., Domingues, A. L. C., Owen, J. S., & de Menezes Lima, V. L. (2014). Human plasma lipid modulation in schistosomiasis mansoni depends on apolipoprotein e polymorphism. *PloS One, 9*(7), e101964.

Deelder, A. M., Kornelis, D., Van Marck, E. A. E., Eveleigh, P. C., & Van Egmond, J. G. (1980). Schistosoma mansoni: Characterization of two circulating polysaccharide antigens and the immunological response to these antigens in mouse, hamster, and human infections. *Experimental Parasitology, 50*(1), 16-32.

De la Torre-Escudero, E., Valero, L., Pérez-Sánchez, R., Manzano-Román, R., & Oleaga, A. (2012). Proteomic identification of endothelial cell surface proteins isolated from the hepatic portal vein of mice infected with Schistosoma bovis. *Journal of proteomics, 77*, 129-143.

Dimenstein, R., Carvalho, V. C., Oliveira, D. N., & Gillett, M. P. (1991). Alterations in the levels

and lipid composition of plasma lipoproteins (VLDL, LDL and HDL) in Brazilian patients with hepatosplenic schistosomiasis mansoni. *Brazilian Journal of Medical and*

Biological Research= Revista Brasileira de Pesquisas Medicas e Biologicas/Sociedade Brasileira de Biofisica., 25(11), 1091-1102.

Doehring, E., Feldmeier, H., & Daffalla, A. A. (1983). Day-to-day variation and circadian rhythm of egg excretion in urinary schistosomiasis in the Sudan. *Annals of Tropical Medicine and Parasitology, 77*(6), 587-594.

Doenhoff, M. J., Stanley, R. G., Griffiths, K., & Jackson, C. L. (2002). An anti-atherogenic effect of Schistosoma mansoni infections in mice associated with a parasite-induced lowering of blood total cholesterol. *Parasitology, 125*(05), 415-421.

Duval, F., Frommherz, K., Atger, V., Drüeke, T., & Lacour, B. (1989). Influence of end-stage renal failure on concentrations of free apolipoprotein A-1 in serum. *Clinical Chemistry, 35*(6), 963-966.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science, 246*(4926), 64-71.

Gomes, L. I., Enk, M. J., & Rabello, A. (2014). Diagnosing schistosomiasis: where are we?. *Revista da Sociedade Brasileira de Medicina Tropical, 47*(1), 3-11.

Graversen, J. H., Castro, G., Kandoussi, A., Nielsen, H., Christensen, E. I., Norden, A., & Moestrup, S. K. (2008). A pivotal role of the human kidney in catabolism of HDL protein components apolipoprotein AI and A-IV but not of A-II. *Lipids, 43*(5), 467-470.

Gryseels, B., Polman, K., Clerinx, J., & Kestens, L. (2006). Human schistosomiasis. *The Lancet, 368*(9541), 1106-1118.

Houmsou, R. S., Amuta, E. U., Wama, B. E., & Hue, T. D. (2013). Proteinuria as a Morbidity Marker of Urinary Schistosomiasis in School Children Living in Onchocerciasis Endemic Areas of Benue State, Nigeria. *International Journal of Life Science and Medical Research, 3*(6), 246.

Issaq, H. J., Veenstra, T. D., Conrads, T. P., & Felschow, D. (2002). The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. *Biochemical and Biophysical Research Communications, 292*(3), 587-592.

Kaysen, G. A. (2009). Lipid and lipoprotein metabolism in chronic kidney disease. *Journal of Renal Nutrition, 19*(1), 73-77.

Lopez, A. D., Mathers, C. D., Ezzati, M., Jamison, D. T., & Murray, C. J. (2006). Global and

regional burden of disease and risk factors, 2001: systematic analysis of population health data. *The Lancet, 367*(9524), 1747-1757.

McManus, D. P., & Loukas, A. (2008). Current status of vaccines for schistosomiasis. *Clinical Microbiology Reviews, 21*(1), 225-242.

Ndao M, Spithill TW, Caffrey R, Li H, Podust VN, Perichon R, Santamaria C, Ache A, Duncan M, Powell MR, Ward BJ, 2010. Identification of novel diagnostic serum biomarkers for Chagas' disease in asymptomatic subjects by mass spectrometric profiling. *Journal of Clinical Microbiology, 48(*4), 1139-1149.

Papadopoulos, M. C., Abel, P. M., Agranoff, D., Stich, A., Tarelli, E., Bell, B. A., ... & Krishna, S. (2004). A novel and accurate diagnostic test for human African trypanosomiasis. *The Lancet, 363*(9418), 1358-1363.

Pirillo, A., Catapano, A. L., & Norata, G. D. (2015). HDL in infectious diseases and sepsis. In *High Density Lipoproteins* (pp. 483-508). Springer International Publishing.

Pullan, R. L., Smith, J. L., Jasrasaria, R., & Brooker, S. J. (2014). Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. *Parasit Vectors, 7*(1), 37.

Rasoamanamihaja, C. F., Rahetilahy, A. M., Ranjatoarivony, B., Dhanani, N., Andriamaro, L., Andrianarisoa, S. H., & Jourdan, P. M. (2016). Baseline prevalence and intensity of schistosomiasis at sentinel sites in Madagascar: Informing a national control strategy. *Parasites & vectors, 9*(1), 1.

Richter, J., Holtfreter, M., Mouahid, G., & Moné, H. (2016). Acute anuria after a family vacation to Corsica/France. Parasitology Research, 1-3.

Ridi, R. E., Tallima, H., Mohamed, S. H., & Montash, M. (2004). Immunology-Depletion of Schistosoma mansoni Lung-Stage Schistosomula Cholesterol by Methyl-b-Cyclodextrin Dramatically Increases Specific Antibody Binding to Surface Membrane Antigens. *Journal of Parasitology, 90*(4), 727-732.

Rioux, M. C., Carmona, C., Acosta, D., Ward, B., Ndao, M., Gibbs, B. F., ... & Spithill, T. W. (2008). Discovery and validation of serum biomarkers expressed over the first twelve weeks of Fasciola hepatica infection in sheep. *International Journal for Parasitology, 38*(1), 123-136.

Roheim, P. S. (1986). Atherosclerosis and lipoprotein metabolism: role of reverse cholesterol

transport. *The American Journal of Cardiology, 57*(5), C3-C10.

Rosser, A., Rollinson, D., Forrest, M., & Webster, B. L. (2015). Isothermal Recombinase Polymerase amplification (RPA) of Schistosoma haematobium DNA and oligochromatographic lateral flow detection. *Parasites & Vectors,8*(1), 1-5.

Sandoval, N., Siles-Lucas, M., Perez-Arellano, J. L., Carranza, C., Puente, S., Lopez-Aban, J., & Muro, A. (2006). A new PCR-based approach for the specific amplification of DNA from different Schistosoma species applicable to human urine samples. *Parasitology, 133*(05), 581-587.

Santamaria C, Chatelain E, Jackson Y, Miao Q, Ward BJ, Chappuis F, Ndao M, 2014. Serum biomarkers predictive of cure in Chagas disease patients after nifurtimox treatment. *BMC Infectious Diseases, 3;14*(1):1.

Santos, J., Chaves, J., Araújo, H., Vale, N., Costa, J. M., Brindley, P. J., ... & Santos, L. L. (2015).

Comparison of findings using ultrasonography and cystoscopy in urogenital schistosomiasis in a public health centre in rural Angola. SAMJ: *South African Medical Journal, 105*(4), 312-315.

Scebba, F., Tognotti, D., Presciuttini, G., Gabellieri, E., Cioni, P., Angeloni, D., ... & Morelli, E. (2016). A SELDI-TOF approach to ecotoxicology: comparative profiling of low molecular weight proteins from a marine diatom exposed to CdSe/ZnS quantum dots. *Ecotoxicology and Environmental Safety, 123*, 45-52.

Silva, C. A. D., Oliveira, K. F. D., Carvalho, V. C. O. D., Domigues, A. L. C., Brandt, C. T., & Lima, V. L. D. M. (2002). Surgical treatment effect on the liver lecitin: cholesterol acyltransferase (LCAT) in schistosomiasis mansoni. *Acta Cirurgica Brasileira,* 17, 28-30.

Soto-Miranda, E., Carreón-Torres, E., Lorenzo, K., Bazán-Salinas, B., García-Sánchez, C., Franco, M., ... & Rodriguez-Pérez, J. M. (2012). Shift of high-density lipoprotein size distribution toward large particles in patients with proteinuria. *Clinica Chimica Acta*, 414, 241-245.

Sprong, H., Suchanek, M., van Dijk, S. M., van Remoortere, A., Klumperman, J., Avram, D., & Thiele, C. (2006). Aberrant receptor-mediated endocytosis of Schistosoma mansoni glycoproteins on host lipoproteins. *PLoS Medicine, 3*(8), e253.

Stothard, J. R., Kabatereine, N. B., Tukahebwa, E. M., Kazibwe, F., Rollinson, D., Mathieson, W. & Fenwick, A. (2006). Use of circulating cathodic antigen (CCA) dipsticks for detection of intestinal and urinary schistosomiasis. *Acta Tropica, 97*(2), 219-228.

Subramanian, A. K., Mungai, P., Ouma, J. H., Magak, P., King, C. H., Mahmoud, A. A., & King, C. L. (1999). Long-term suppression of adult bladder morbidity and severe hydronephrosis following selective population chemotherapy for Schistosoma haematobium. *The American Journal of Tropical Medicine and Hygiene, 61*(3), 476-481.

Tallima, H.& El Ridi, R. (2005) Methyl-beta-Cyclodextrin treatment and filipin staining reveal the role of cholesterol in surface membrane antigen sequestration of Schistosoma mansoni and S. haematobium lung-stage larvae. *Journal of Parasitology 91,* 720–725.

Van Dam, G. J., Wichers, J. H., Ferreira, T. F., Ghati, D., Van Amerongen, A., & Deelder, A. M. (2004). Diagnosis of schistosomiasis by reagent strip test for detection of circulating cathodic antigen. *Journal of Clinical Microbiology, 42*(12), 5458-5461.

Van Lieshout, L., Polderman, A. M., & Deelder, A. M. (2000). Immunodiagnosis of schistosomiasis by determination of the circulating antigens CAA and CCA, in particular in individuals with recent or light infections. *Acta Tropica, 77*(1), 69-80.

Vokřál, I., jedličková,., Jirasko, R., Stuchlikova, L., Bartikova, H., Skalova, L., ... & Szotakova, B. (2013). The metabolic fate of ivermectin in host (Ovis aries) and parasite (Haemonchus contortus). *Parasitology, 140*(03), 361-367.

Wang, L. D., Guo, J. G., Wu, X. H., Chen, H. G., Wang, T. P., Zhu, S. P., ... & Zhou, X. N. (2009). China's new strategy to block Schistosoma japonicum transmission: experiences and impact beyond schistosomiasis. *Tropical Medicine & International Health, 14*(12), 1475-1483.

World Health Organization. (1991). Basic laboratory methods in medical parasitology.

World Health Organization (2012). Accelerating work to overcome the global impact of neglected tropical diseases. A roadmap for implementation.

Yui, Y., Aoyama, T., Morishita, H., Takahashi, M., Takatsu, Y., & Kawai, C. (1988). Serum prostacyclin stabilizing factor is identical to apolipoprotein AI (Apo AI). A novel function of Apo AI. *Journal of Clinical Investigation, 82*(3), 803.

Zuo, X., Echan, L., Hembach, P., Tang, H. Y., Speicher, K. D., Santoli, D., & Speicher, D. W.

(2001). Towards global analysis of mammalian proteomes using sample prefractionation prior to narrow pH range two-dimensional gels and using one-dimensional gels for insoluble and large proteins. *Electrophoresis, 22*(9), 1603-1615.

Zuo, X., Hembach, P., Echan, L., & Speicher, D. W. (2002). Enhanced analysis of human breast cancer proteomes using micro-scale solution isoelectrofocusing combined with high resolution 1-D and 2-D gels. *Journal of Chromatography B, 782*(1), 253-265.

Zuo, X., & Speicher, D. W. (2002). Comprehensive analysis of complex proteomes using microscale solution isoelectrofocusing prior to narrow pH range two-dimensional electrophoresis. *Proteomics, 2*(1), 58-68.

CHAPTER V

GENERAL DISCUSSION

Schistosomiasis is a chronic debilitating disease that affects ~ 200 million people in more than 70 countries. Despite the wide availability and relatively low cost of praziquantel, treatment is often delayed or omitted altogether because the available diagnostic tests have low sensitivity both during early and late disease stages and re-infection is common. Therefore, for praziquantel to be effectively used to reduce morbidity, non-invasive and reliable diagnostic assays that can detect early and late stages of the disease need to be developed.

One of the available diagnostic assays is the Kato-Katz technique. This technique, although widely used, has low sensitivity due to the widespread application of chemotherapy which results in low parasite burdens. In addition, it is less efficient in low transmission areas and after treatment (Enk et al., 2010; Grenfell et al., 2012). The result of this method is influenced by numerous factors, such as the intensity of infection, the timing of egg excretion, and egg distribution in the stool. Repeated fecal examinations can increase sensitivity, but reduces patient compliance, which limits the effectiveness of this technique. The other disadvantage of this detection method is that it is time consuming, which makes it unsuitable for large-scale epidemiological surveys (Wang et al., 2015). Other diagnostic methods that detect antibodies or antigens in serum and/or urine samples are also available (Coulibaly et al., 2013; Deelder et al., 1993; Doenhoff et al., 2004; Grenfell et al., 2012). Antibody detection methods have a relatively high sensitivity but low specificity and cannot distinguish between current and past infection. On the other hand, a growing body of evidence suggests that antigen detection methods are more promising than either direct (eg: eggs) or indirect (antibodies) parasitological methods (Colley et al., 2013; Foo et al., 2015; Van Dam et al., 2013). Parasite antigen detection

may indicate the presence of active infection and reduce the rate of false-positive results compared to antibody detection methods. Therefore, antigen detection improves diagnosis accuracy and reduces the cost of field investigations (Stothard, 2009; Worrell et al., 2015).

To overcome the limitations of these various techniques, we need to introduce new diagnostic approaches through the selection of biomarkers that can be used for the detection of schistosomiasis patients. Biomarkers are considered as biological factors that could provide early diagnosis of disease and are also biochemical monitors for the response of patients to treatment. This makes biomarkers an ideal diagnostic tool. This approach may (a) help avoid false-positive or false-negatives; (b) assist in the detection of different stages of infection such as early, acute and chronic stages; and (c) distinguishes between active disease and past infection.

Despite the significant improvement in the understanding of basic biology of schistosomiasis, the complexity of the parasite itself and its interactions with the vertebrate host have led to serious difficulties in making an accurate diagnosis which, in turn, has made it more difficult to eradicate the disease. Schistosomes are multi-stage parasites and in each stage they inhabit a different anatomical location inside the host. Because of this, they potentially express different protein profiles. Therefore, a more complete understanding of protein expression profiles may be very useful to detect each of their life stages. Experimental schistosomal infection in laboratory animals has contributed significantly to understanding the immunobiology of infection; particularly the mechanisms of egg-induced granuloma and fibrosis (Abath et al., 2006). The experimental animals can be infected with a known number of cercariae. After the experimental animals are infected one can compare the protein profiles of infected animals to those of non-infected animals. Rodents, mainly inbred mice, tend to be the animals of choice because of their availability and susceptibility to infection (Boros &Whitfield, 1999). Studies

using experimental animals of schistosomiasis need to be validated in humans. Caution is required when interpreting results from mouse models because the infection is not the same as the clinical situation. In the clinical situation, there are potentially confounding factors such as co-infections, re-infections, host and parasite genetics, and environmental factors that cannot be accurately evaluated (Abath et al., 2006; Fung et al., 2014).

Nevertheless, proteomic analysis of serum proteins in diseased and control mouse models allowed us to uncover a number of potential disease biomarkers.

In the present thesis, I used several proteomic approaches to identify either parasite or host-derived products (i.e., biomarkers) related to different stages of schistosome infection. The rationale for using these approaches in potential diagnostic tests for the disease was because less invasive methods of detection are favoured. I chose to examine the presence of biomarkers in biological fluid such as serum, because it is considered a valuable source for biomarker discovery (Srinivas et al., 2001). Several investigators have attempted to identify biomarkers in serum that reflect specific pathological states. Studying the serum proteome emphasizes differences in protein expression, reflects certain pathophysiological states, and provides useful information about disease diagnosis, prognosis or treatment response (Ahmed, 2008). We chose serum because of the fact that schistosomes' antigens may be liberated as a result of parasite turnover and reaches the blood. However, serum is very complex with relatively large amounts of the most abundant proteins that can interfere with the identification of more interesting, disease-specific and low-abundance proteins (Anderson & Anderson, 2002; Tirumalai et al., 2003). To detect the low abundance proteins, fractionation of the proteome is required (Hanash et al., 2008).

To remove these highly abundant proteins, I used isoelectric focusing fractionation (IEF) and ion-exchange fractionations to uncover the low abundance proteins. IEF is accomplished by loading the protein sample into connected five ZOOM® IEF chambers separated by disks that contain buffers of defined pH. The result is a significant reduction in sample complexity in the form of a set of five highly resolved protein fractions suitable for further analysis by 1-DE. The proteins were focused using a program of progressively increasing voltage (100 V for 20 min, 200 V for 80 min, 600 V for 80 min). IEF offers several advantages for peptide analysis such as a wide range of loading capacity, superior resolution, and good reproducibility (Hörth et al., 2006; Hubner et al., 2008; Lam et al., 2007). It has been reported that solution-IEF has high reproducibility and efficient resolution compared to ion exchange (Slebos et al., 2008). However, post-IEF sample processing is considered a limitation as it involves the excision of the gel strip into sections, followed by extracting and cleaning up of peptides from these gel pieces. In addition, it is difficult to predict the actual amount of peptides or proteins in each compartment of the IEF solution (Hörth et al., 2006). The major problem we faced with this technique was that we had to use 200 µl of each sample and this took a long time to process. To overcome this problem, we used a pool from each group. However, sample pooling led to an unavoidable loss of information. Therefore, we used ion-exchange fractionation in which the samples were separated into six fractions within 20 min by step pH elution (buffers of pH 3–9, and an organic isopropanol-based buffer) by ion exchange chromatography. This allowed us to use small amounts of samples (20 µl) and was very fast in comparison to IEF.

We have demonstrated that SELDI mass spectrometry can be employed for rapid analysis of a large numbers of samples and can generate patterns of differentially expressed proteins that may distinguish disease states from controls as well as differentiate different stages of the disease

in experimental animals. Remarkably, using SELDI-TOF MS, a limited sample preparation is needed, this technique is a high-throughput one and it is ideally suited for low MW proteins (<20 kDa) profiling (Tang et al., 2004). The advantage of using SELDI-TOF MS in our experiments is that it is a hybrid technology that permits the separation of the proteins (by solid-phase chromatography) and analysis (by mass spectrometry) to be performed using the same analyzer. Therefore, the protein profiles of samples can be rapidly compared (Gretzer et al., 2003; Shi et al., 2009). The SELDI platform is association with protein chip arrays. The surfaces of these chips are coated with a protein-fractionating resin (Issaq et al., 2002 and Merchant and Weinberger, 2000). These chip arrays have a detection limit for any individual compound for a given set of experimental variables (Tang et al., 2004). To overcome this limitation, we used three different kinds of chip arrays; immobilized metal affinity capture (IMAC), weak-cation exchange (CM10) and reverse phase (H50) Protein-Chip™ arrays. The limitation of using SELDI-TOF MS is that it has a poor reproducibility. Therefore, it is very important to standardize the pre-analytic and analytic factors (Banks, 2008; Bruegel et al., 2009; Callesen et al., 2009). As a result, in our study we applied strict Standard Operating Procedures to improve data quality and reproducibility. The study subjects were carefully selected and the preanalytical processes such as study design, sample collection and processing were carefully determined (Rai & Vitzthum 2006). The samples were kept in -80 °C because most of proteins in body fluids are stable for a long time at -80 °C. Samples were aliquoted into smaller volumes to avoid repeated freeze–thaw cycles. Plasma or serum samples could be exposed to less than five freeze/thaw cycles before significant deterioration in quality of protein profile is observed (Caffrey, 2010; Tammen et al., 2005). Technical replicates have been used within the same samples (Hong et al., 2005). We also used a robotics system and a defined matrix drying time (Aivado et al., 2005;

Schaub et al., 2004). It is known that the mass-axis of the SELDI output shifts from experiment to experiment by up to 0.1–0.2% of the mass/charge value (Yasui et al., 2003). We then account for a shifting (measurement error of ± 5% mass range) problem of the x axis in SELDI output in our experiments. In spite of these limitations, SELDI has been successfully applied in the present studies.

Three independent studies have presented specific serum biomarkers associated with the schistosomiasis infection using the SELDI-MS platform. In the first study, we studied serum protein expression patterns in groups of mice infected with different numbers of cercariae, 66 candidate biomarkers were detected. Used in various combinations, these biomarkers could reliably diagnose early-stage disease as well as distinguish between acute and chronic infection. Additionally, they could diagnose *S. mansoni* infection regardless of the number of cercariae used for infection.

A classification tree was established by Biomarker Pattern Software using just one candidate biomarker peak to distinguish between acutely-infected animals and controls. This single biomarker decision tree achieved 100% sensitivity and 100% specificity (m/z 46.1 kDa).

The second study were able to detect 74 possible biomarkers based simply on m/z ratios with a high diagnostic prediction, allowing discrimination between the uninfected and infected serum proteomes.

In the third study, we studied serum protein expression patterns in 85 human samples (55 negative controls, 30 *S. haematobium*-infected). A total of 82 candidate biomarkers for the comparison between controls vs. *S. haematobium* were detected.

However, a major disadvantage of SELDI-TOF MS technology is its inability to identify proteins. The only information that SELDI provides is the m/z value of the potential biomarker

protein. Characterizing proteins or peptides by their m/z value is not enough for diagnosis because their unknown identity will hinder additional validation with other methods (Tambor et al., 2010). This is why this technology might be replaced in the near future with the shotgun proteomics technology. Once identification of the potential biomarker protein is achieved and suitable antibodies developed against these biomarkers, SELDI-identified biomarkers will combine the great accuracy of SELDI with the much needed practical aspects of immunoassays such as ELISA.

To identify new biomarkers, we used other MS platforms such as MALDI-TOF, ESI and Velos Orbitrap. As a result, we were able to identify many potential biomarkers in the sera of infected human and mice. The MALDI-TOF MS approach yielded eight differentially-expressed host proteins in the serum of infected mice at different disease stages. Among the host proteins intriguingly identified by MALDI were transferrin and alpha 1-antitrypsin in (acute/chronic) disease. These two proteins were identified with 12 and 4 peptides respectively, while all the other identified proteins were identified with only one peptide and their up-regulation was confirmed by Western blot. Then we used two different MS platforms to study differentially-expressed host proteins in the serum of infected patients and mice. For human samples we used ESI and for mice samples we used Orbitrap. Sequencing with ESI of human samples revealed one proposed protein for the band found in in *S. haematobium* infected sera. Apolipoprotein A-I, was revealed at 28 kDa band, which was confirmed by Western blot and ELISA.

One major advantage of MALDI over ESI is that singly charged ions are detected for the analyte, while ESI generates multiple charged ions for higher molecular weight molecules that complicate the generated mass spectra (Mesri, 2014).

Mass spectrometry has been gaining importance and it has been making a gradual progress until recently, when instruments such as Orbitrap are able to achieve mass resolutions of hundreds of thousands of units with a wide dynamic range. Such values are rarely available for MALDI MS instruments, Orbitrap have certain qualities that make it advantageous for advanced analysis. In the first study, Velos Orbitrap MS identified ~200 parasite-origin proteins in the serum during the acute stage, ~105 parasite-origin proteins during the chronic stage and ~296 and ~220 differentially-expressed host-proteins identified in the serum during the acute and chronic stages respectively. We identified a large number of parasite-origin proteins in serum. Van Diepen et al., (2012) reported that a relatively small number of schistosome antigens are exposed to the host in the tegument and more antigens become exposed after the parasite dies. Therefore, it would be beneficial to identify a circulating antigen in host sera to diagnose schistosomiasis (Wang et al., 2015). Proteins identified by Orbitrap were possibly shed by schistosomula, released from dying worms, released by damaged tegumental cell layer, or were secreted/excreted by the worms (Francis & Bickle, 1992; Jankovic et al., 1996; Mohamed et al., 1998). The Orbitrap data also suggested the presence of schistosome (25 kDa) Glutathione S-transferase (GST) that was confirmed by Western blot. Most of the identified proteins are identified only by a single peptide match (single-hit proteins). This may be due to many factors: low concentration of parasite antigens in the serum of the host, few tryptic peptides in small proteins, or masking by other more expressed proteins (Higdon & Kolker, 2007). The problem with ignoring these proteins will result in the loss of potential biomarkers. GST was one of the proteins which identified with only one peptide and we proved its presence by Western blot.

The major limitation that could hinder the clinical application of peptide/protein biomarkers is the difficulty encountered when producing certain antibodies for sensitive clinical

tests such as immunoassays. They are very sensitive, and they can detect protein concentrations in the pg/ml range with good specificity, if an antibody with the desired performance is available. However, immunoassays have limitations (Scherl, 2015). Antibodies recognize a polypeptide epitope of typically eight to seventeen amino acids. Therefore, an antibody might detect a common portion of a protein present in different proteoforms (Smith, & Kelleher, 2013). ELISA is considered the gold standard for several clinical applications. Unfortunately, commercial assay kits are not available for many of the proteins that were of interest to us. Additionally, antibody production for a large number of peptide candidates is costly and time-consuming. Recently, the progress in mass spectrometry (Anderson & Hunter, 2006; Janecki et al., 2007) might provide an alternative to ELISA that can accurately detect several peptides. The recent advance in mass spectrometry-based targeted protein quantification affords a powerful approach to systematically and quantitatively measure quantitative differences in protein profiles of different samples. The quantitative tool depends on using a synthetic stable isotope labeled peptide or protein which is similar to the analyzed peptide or protein and acts as an internal standard to quantify the corresponding candidate protein (Pan et al., 2008). Recently, selected reaction monitoring-mass spectrometry (SRM-MS), also called multiple reaction monitoring (MRM), can also be used for biomarker validation (Addona et al., 2009; Grote et al., 2013). SRM-MS has emerged as a potentially useful technique for targeted multiplexed quantitative proteomics to monitor and quantify proteins in plasma samples. In this technique, two mass analyzers are used as static mass filters to monitor a particular fragment ion of a selected precursor ion. Multiple SRM transitions can be measured on the chromatographic time scale by alternating between the different precursor/fragment pairs within the same experiment. Typically, the triple quadrupole instrument cycles through a series of transitions and records the signal of

each transition as a function of the elution time (Mesri, 2014). Furthermore, SRM can be performed to build a calibration curve that can offer an absolute quantification of the native peptide and subsequently its parent protein (Paczesny et al., 2013; Ota et al., 2015).

In the second study, we continued our investigation of sera in *S. mansoni* infected mice using Orbitrap. We identified 239 differentially-expressed proteins from the sera of the acutely- and chronically-infected mice compared to controls. Twenty-two proteins were up-regulated in infected mice (acutely and chronically) compared to controls. Eight of these were up-regulated in both the acute and chronic stages compared to controls. We identified nineteen proteins in control samples with higher PIP compared to infected samples (acutely or chronically). Nine of these were down-regulated in both acute and chronic stages compared to controls. By comparing the molecular weights of the seventeen proteins that were consistently up- or down-regulated in schistosomiasis samples versus controls to query the mouse SELDI-TOF MS database for the corresponding peaks. Four distinct SELDI peaks that differed substantially between infected and control sera were matched. We also looked in our SELDI human samples data for the same seventeen proteins and we found only one peak at 28 kDa value that corresponded to carbonic anhydrase 1 (CA1). CA1 has a theoretical molecular weight of 28 kDa which corresponded well with one of the selected SELDI peaks in mice and human samples. We identified a decreased serum level of CA1 and this is a novel biomarker in serum of *S. mansoni*-infected mice and humans. This would imply that this biomarker might be considered as a very suitable diagnostic marker.

By means of a well-established murine model of schistosomiasis it was confirmed that CA1 could be down-regulated in serum during acute and chronic stages of the disease. We continued a proof-of-concept study on available patients with schistosomiasis. In a first

approach, we showed that CA1 could be down-regulated in all of eight chronically-infected patients by Western blot. Followed by measuring serum concentrations of CA1 in 17 human subjects with chronic schistosomiasis, they were significantly lower than levels in 17 normal controls (n=17) by ELISA. This intriguing observation might be related to colonic inflammation due to schistosomiasis-induced reactions that are associated with the accumulation of schistosome eggs or, alternatively, to changes in zinc serum levels. Studies are planned to determine if serum CA1 levels return to normal after praziquantel therapy and the kinetics of such normalization. We are also curious to know if serum CA1 levels are reduced in subjects with other infectious and non-infectious conditions that cause bowel inflammation. We also want to determine the possible contribution of zinc deficiency to lower CA1 levels. There are no previous clinical studies measuring the level of CA1 in schistosomiasis. It would seem worthy to further validate CA1 as a candidate biomarker of schistosomiasis. However, the inclusion of additional clinical groups such as other helminth and protozoan infections would be required to determine the specificity of this candidate biomarker.

This study does not provide a protocol intended for direct transfer into clinical application. Nevertheless, the detection and quantification of CA1 from serum might carry the potential of becoming a novel diagnostic tool for schistosomiasis. Due to the small number of available patients, this finding clearly awaits confirmation in larger studies. We suggest that future work includes more samples, collection of serum samples from patients and controls. Sample history should be taken into consideration. Some of the things that need to be taken into consideration are storage information of the samples, diseases stage, medication, pathology, age, gender and patient's condition. This distribution should be as constant as possible in all the collected samples, and it should characterise the precise disease/healthy state. Otherwise,

normalising the analytical results becomes problematic. Lawton et al., (2008) studied the effects of age, sex and race on plasma metabolites. In their study, the patients were Caucasian, African-American and Hispanic with a 20 to 65 age range. They found that more than 100 metabolites were associated with age with smaller numbers associated with sex or race. The samples should be collected in freezer-type tubes, immediately snap frozen and stored in a freezer until analyzed.

Further studies are needed to evaluate the application of these putative biomarkers in diagnostic tests. Our study confirms that MS-based proteomics is a powerful approach in biomarker discovery of infectious diseases in humans. However, to obtain a more comprehensive picture it would have been advantageous if we had analyzed a larger number of samples obtained from different Schistosoma species, as well as samples from other helminth and protozoan infections. Our study is only a first step and further verification and validation of the identified biomarkers is needed.

SUMMARY AND CONCLUSIONS

The discovery of novel biomarkers is crucial for the early detection of the disease and characterization of its progression. Recent advances in MS technologies allow large-scale analysis of proteins. This could be used to compare the protein profiles of schistosomiasis-infected and control serum samples. The selection of the sample set is one of the first issues that one will encounter in such studies. After that, candidate protein markers must be assessed by a targeted assay and validated in large independent cohorts. This validation step will allow candidate biomarkers to be transferred to clinical use.

Schistosomiasis was an interesting disease to study, due to its high prevalence. Biomarkers for schistosomiasis are currently not well-characterised. In this research, we examined the differences between diseased and healthy serum samples in order to identify

biomarkers. In this work a mouse model was used, and the effect of using different numbers of the infective larvae for infection as well as the time of infection on the serum protein profiles was also investigated. The outcome of this work was that several proteins were found to be differentially regulated between diseased and healthy humans and animals.

Just because protein can be detected, this does not necessarily reveal whether or not it could be a potential diagnostic target. This type of functional analysis for putative biomarkers will continue to need to be assessed.

Future prospective of biomarkers in schistosomiasis

The identification of biomarkers could improve treatment strategies in schistosomiasis and lead to targeted therapies. The use of biomarkers might also decrease the economic burden encountered when treating the disease. Moreover, an automated and inexpensive standardized biomarker is necessary for detection in schistosomiasis. Unfortunately, most schistosomiasis cases can only be diagnosed after egg deposition. Thus, by frequently evaluating serum biomarkers, changes can be easily found during early stages of infection. Therefore, further improvement in current schistosomiasis biomarker screening assays is needed.

References

Abath, F. G., Morais, C. N., Montenegro, C. E. L., Wynn, T. A., & Montenegro, S. M. (2006). Immunopathogenic mechanisms in schistosomiasis: what can be learnt from human studies?. *Trends in Parasitology, 22*(2), 85-91.

Ahmed, F. E. (2008). Utility of mass spectrometry for proteome ana lysis: part I. Conceptual and experimental approaches.

Addona, T. A., Abbatiello, S. E., Schilling, B., Skates, S. J., Mani, D. R., Bunk, D. M., ... & Carr, S. A. (2009). Multi-site assessment of the precision and reproducibility of multiple reaction monitoring–based measurements of proteins in plasma. *Nature Biotechnology, 27*(7), 633-641.

Anderson, N. L., & Anderson, N. G. (2002). The human plasma proteome history, character, and diagnostic prospects. *Molecular & Cellular proteomics, 1*(11), 845-867.

Anderson, L., & Hunter, C. L. (2006). Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Molecular & Cellular Proteomics, 5*(4), 573-588.

Aivado, M., Spentzos, D., Alterovitz, G., Otu, H. H., Grall, F., Giagounidis, A. A., ... & Libermann, T. A. (2005). Optimization and evaluation of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) with reversed-phase protein arrays for protein profiling. *Clinical Chemical Laboratory Medicine, 43*(2), 133-140.

Banks, R. E. (2008). Preanalytical influences in clinical proteomic studies: raising awareness of fundamental issues in sample banking. *Clinical Chemistry, 54*(1), 6-7.

Boros, D. L. (1999). T helper cell populations, cytokine dynamics, and pathology of the schistosome egg granuloma. *Microbes and Infection, 1*(7), 511-516.

Bruegel, M., Planert, M., Baumann, S., Focke, A., Bergh, F. T., Leichtle, A., ... & Fiedler, G. M. (2009). Standardized peptidome profiling of human cerebrospinal fluid by magnetic bead separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Proteomics, 72*(4), 608-615.

Caffrey, R. E. (2010). A review of experimental design best practices for proteomics based biomarker discovery: focus on SELDI-TOF. *The Urinary Proteome: Methods and Protocols,* 167-183.

Callesen, A. K., Madsen, J. S., Vach, W., Kruse, T. A., Mogensen, O., & Jensen, O. N. (2009). Serum protein profiling by solid phase extraction and mass spectrometry: A future diagnostics tool?. *Proteomics, 9*(6), 1428-1441.

Coulibaly, J. T., N'Gbesso, Y. K., Knopp, S., N'Guessan, N. A., Silué, K. D., van Dam, G. J., ... & Utzinger, J. (2013). Accuracy of urine circulating cathodic antigen test for the diagnosis of Schistosoma mansoni in preschool-aged children before and after treatment. *PLoS Neglected Tropical Diseases, 7*(3), e2109.

Colley, D. G., Binder, S., Campbell, C., King, C. H., Tchuenté, L. A. T., N'Goran, E. K., ... & Rathbun, S. (2013). A five-country evaluation of a point-of-care circulating cathodic antigen urine assay for the prevalence of Schistosoma mansoni. *The American Journal of Tropical Medicine and Hygiene, 88*(3), 426-432.

Deelder, A. M., Qian, Z. L., Kremsner, P. G., Acosta, L., Rabello, A. L., Enyong, P., ... & Rotmans, J. P. (1993). Quantitative diagnosis of Schistosoma infections by measurement of circulating antigens in serum and urine. *Tropical and Geographical Medicine, 46*(4), 233-238.

Doenhoff, M. J., Chiodini, P. L., & Hamilton, J. V. (2004). Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies?. *Trends in Parasitology*, *20*(1), 35-39.

Enk, M. J., Amaral, G. L., Silveira-Lemos, D., Teixeira-Carvalho, A., Martins-Filho, O. A., Correa-Oliveira, R., ... & Massara, C. L. (2010). Rural tourism: a risk factor for schistosomiasis transmission in Brazil. *Memórias do Instituto Oswaldo Cruz, 105*(4), 537-540.

Foo, K. T., Blackstock, A. J., Ochola, E. A., Matete, D. O., Mwinzi, P. N., Montgomery, S. P., ... & Secor, W. E. (2015). Evaluation of Point-of-Contact Circulating Cathodic Antigen Assays for the Detection of Schistosoma mansoni Infection in Low-, Moderate-, and High-Prevalence Schools in Western Kenya. *The American Journal of Tropical Medicine and Hygiene,* 14-0643.

Francis, P., & Bickle, Q., (1992). Cloning of a 21.7-kDa vaccine-dominant antigengene of Schistosoma mansoni reveals an EF hand-like motif. *Mol BiochemParasitol 50*, 215–224

Fung, K. Y., Nice, E., Priebe, I., Belobrajdic, D., Phatak, A., Purins, L., ... & Cosgrove, L. (

2014). Colorectal cancer biomarkers: To be or not to be? Cautionary tales from a road well travelled. *World Journal of Gastroenterology: WJG, 20*(4), 888.

Grenfell, R. F. Q., Silva-Moraes, V., Taboada, D., de Mattos, A. C. A., de Castro, A. K. S., & Coelho, P. M. Z. (2012). Immunodiagnostic methods: what is their role in areas of low endemicity? *The Scientific World Journal*, *2012*.

Gretzer, M. B., Partin, A. W., Chan, D. W., & Veltri, R. W. (2003). Modern tumor marker discovery in urology: surface enhanced laser desorption and ionization (SELDI*). Reviews in Urology, 5*(2), 81.

Grote, E., Fu, Q., Ji, W., Liu, X., & Van Eyk, J. E. (2013). Using pure protein to build a multiple reaction monitoring mass spectrometry assay for targeted detection and quantitation. Heart Proteomics: *Methods and Protocols*, 199-213.

Hanash, S. M., Pitteri, S. J., & Faca, V. M. (2008). Mining the plasma proteome for cancer biomarkers. *Nature, 452*(7187), 571-579.

Hong, H., Dragan, Y., Epstein, J., Teitel, C., Chen, B., Xie, Q., ... & Tong, W. (2005). Quality control and quality assessment of data from surface-enhanced laser desorption/ionization (SELDI) time-of flight (TOF) mass spectrometry (MS). BMC bioinformatics, 6(Suppl 2), S5.

Higdon, R., & Kolker, E. (2007). A predictive model for identifying proteins by a single peptide match. *Bioinformatics, 23*(3), 277-280.

Hörth, P., Miller, C. A., Preckel, T., & Wenz, C. (2006). Efficient fractionation and improved protein identification by peptide OFFGEL electrophoresis. *Molecular & Cellular Proteomics, 5*(10), 1968-1974.

Hubner, N. C., Ren, S., & Mann, M. (2008). Peptide separation with immobilized pI strips is an attractive alternative to in-gel protein digestion for proteome analysis. *Proteomics, 8*(23-24), 4862-4872.

Issaq, H. J., Veenstra, T. D., Conrads, T. P., & Felschow, D. (2002). The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. *Biochemical and Biophysical Research Communications, 292*(3), 587-592.

Janecki, D. J., Bemis, K. G., Tegeler, T. J., Sanghani, P. C., Zhai, L., Hurley, T. D., Bosron, W.

F. & Wang, M. (2007). A multiple reaction monitoring method for absolute quantification of the human liver alcohol dehydrogenase ADH1C1 isoenzyme. *Analytical Biochemistry, 369*(1), 18-26.

Jankovic, D., Aslund, L., Oswald, I. P., Caspar, P., Champion, C., Pearce, E., ... & James, S. L. (1996). Calpain is the target antigen of a Th1 clone that transfers protective immunity against Schistosoma mansoni. *The Journal of Immunology, 157*(2), 806-814.

Lawton, K. A., Berger, A., Mitchell, M., Milgram, K. E., Evans, A. M., Guo, L., ... & Milburn, M. V. (2008). Analysis of the adult human plasma metabolome.

Lam, H. T., Josserand, J., Lion, N., & Girault, H. H. (2007). Modeling the isoelectric focusing of peptides in an OFFGEL multicompartment cell. *Journal of Proteome Research, 6*(5), 1666-1676.

Merchant, M., & Weinberger, S. R. (2000). Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. *Electrophoresis, 21*(6), 1164-1177.

Mesri, M. (2014). Advances in Proteomic Technologies and Its Contribution to the Field of Cancer. *Advances in Medicine*, 2014.

Mohamed, M. M., Shalaby, K. A., LoVerde, P. T., & Karim, A. M. (1998). Characterization of Sm20. 8, a member of a family of schistosome tegumental antigens. *Molecular and Biochemical Parasitology, 96*(1), 15-25.

Ota, A., Kovary, K. M., Wu, O. H., Ahrends, R., Shen, W. J., Costa, M. J., ... & Teruel, M. N. (2015). Using SRM-MS to quantify nuclear protein abundance differences between adipose tissue depots of insulin-resistant mice. *Journal of Lipid Research, 56*(5), 1068-1078.

Paczesny, S., Raiker, N., Brooks, S., & Mumaw, C. (2013). Graft-versus-host disease biomarkers: omics and personalized medicine. *International Journal of Hematology, 98*(3), 275-292.

Pan, S., Aebersold, R., Chen, R., Rush, J., Goodlett, D. R., McIntosh, M. W., ... & Brentnall, T. A. (2008). Mass spectrometry based targeted protein quantification: methods and applications. *Journal of Proteome Research, 8*(2), 787-797.

Rai, A. J., & Vitzthum, F. (2006). Effects of preanalytical variables on peptide and protein

measurements in human serum and plasma: implications for clinical proteomics. *Expert review of proteomics, 3*(4), 409-426.

Schaub, S., Wilkins, J., Weiler, T., Sangster, K., Rush, D., & Nickerson, P. (2004). Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Kidney International, 65*(1), 323-332.

Scherl, A. (2015). Clinical protein mass spectrometry. *Methods*. 81, 3-14.

Shi, M., Caudle, W. M., & Zhang, J. (2009). Biomarker discovery in neurodegenerative diseases: a proteomic approach. *Neurobiology of Disease, 35*(2), 157-164.

Slebos, R. J., Brock, J. W., Winters, N. F., Stuart, S. R., Martinez, M. A., Li, M., ... & Liebler, D. C. (2008). Evaluation of strong cation exchange versus isoelectric focusing of peptides for multidimensional liquid chromatography-tandem mass spectrometry. *Journal of Proteome research, 7*(12), 5286-5294.

Smith, L. M., & Kelleher, N. L. (2013). Proteoform: a single term describing protein complexity. *Nature Methods, 10*(3), 186-187.

Srinivas, P. R., Kramer, B. S., & Srivastava, S. (2001). Trends in biomarker research for cancer detection. *The Lancet Oncology, 2*(11), 698-704.

Stothard, J. R. (2009). Improving control of African schistosomiasis: towards effective use of rapid diagnostic tests within an appropriate disease surveillance model. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 103*(4), 325-332.

Tambor, V., Fucikova, A., Lenco, J., Kacerovsky, M., Rehacek, V., Stulik, J., & Pudil, R. (2010). Application of proteomics in biomarker discovery: a primer for the clinician. *Physiol Res, 59*(4), 471-497.

Tammen, H., Schulte, I., Hess, R., Menzel, C., Kellmann, M., Mohring, T., & Schulz-Knappe, P. (2005). Peptidomic analysis of human blood specimens: comparison between plasma specimens and serum by differential peptide display. *Proteomics, 5*(13), 3414-3422.

Tang, N., Tornatore, P., & Weinberger, S. R. (2004). Current developments in SELDI affinity technology. *Mass Spectrometry Reviews, 23*(1), 34-44.

Tirumalai, R. S., Chan, K. C., Prieto, D. A., Issaq, H. J., Conrads, T. P., & Veenstra, T. D. (2003). Characterization of the low molecular weight human serum proteome. *Molecular & Cellular Proteomics, 2*(10), 1096-1103.

Van Dam, G. J., de Dood, C. J., Lewis, M., Deelder, A. M., van Lieshout, L., Tanke, H. J., ... &

Corstjens, P. L. (2013). A robust dry reagent lateral flow assay for diagnosis of active schistosomiasis by detection of< i> Schistosoma</i> circulating anodic antigen. *Experimental Parasitology, 135*(2), 274-282.

Van Diepen, A., van der Velden, N. S., Smit, C. H., Meevissen, M. H., & Hokke, C. H. (2012). Parasite glycans and antibody-mediated immune responses in Schistosoma infection. Parasitology, 139(09), 1219-1230.

Wang, J., Ke, X., Yu, C., & Zhang, J. (2015). Assessment of the diagnostic efficacy of enolase as an indication. *Parasitology research, 115*(1), 151-164.

Worrell, C. M., Bartoces, M., Karanja, D. M., Ochola, E. A., Matete, D. O., Mwinzi, P. N., ... & Secor, W. E. (2015). Cost Analysis of Tests for the Detection of Schistosoma mansoni Infection in Children in Western Kenya. *The American Journal of Tropical Medicine and Hygiene,* 14-0644.

Yasui, Y., Pepe, M., Thompson, M. L., Adam, B. L., Wright, G. L., Qu, Y., ... & Feng, Z. (2003). A data-analytic strategy for protein biomarker discovery: profiling of high-dimensional proteomic data for cancer detection. *Biostatistics, 4*(3), 449-463.

Appendix

Manuscript I

Supplemental table 1

Identified Proteins (200)	Accession Number	Molecular Weight	Identification propability
AF418550_1 albumin precursor [Schistosoma mansoni]	15808978	68 kDa	100%
family S9 non-peptidase homologue (S09 family) [Schistosoma mansoni]	256077324	126 kDa	95%
receptor tyrosine phosphatase type r2a [Schistosoma mansoni]	256078184	267 kDa	95%
nAChR subunit [Schistosoma mansoni]	256077733 (+1)	66 kDa	95%
aminopeptidase P1 (M24 family) [Schistosoma mansoni]	256080084	59 kDa	95%
ryanodine receptor related [Schistosoma mansoni]	256085300	559 kDa	95%
SJCHGC07397 protein [Schistosoma japonicum]	76157105	13 kDa	95%
hypothetical protein [Schistosoma mansoni]	256058663	86 kDa	100%
unknown [Schistosoma japonicum]	56754341 (+1)	40 kDa	100%
hypothetical protein [Schistosoma mansoni]	256076304	338 kDa	95%
hypothetical protein [Schistosoma mansoni]	256087454	328 kDa	99%
vesicular amine transporter [Schistosoma mansoni]	256070152	158 kDa	98%
hypothetical protein [Schistosoma mansoni]	256084562	77 kDa	95%
ell related [Schistosoma mansoni]	256052881 (+1)	93 kDa	95%
SJCHGC08776 protein [Schistosoma japonicum]	76152796	22 kDa	95%
SJCHGC02516 protein [Schistosoma japonicum]	76154880	55 kDa	95%
SJCHGC09815 protein [Schistosoma japonicum]	60701195	10 kDa	95%
hypothetical protein [Schistosoma mansoni]	256048394 (+1)	30 kDa	95%
hypothetical protein [Schistosoma mansoni]	256072930	40 kDa	95%
cell division control protein [Schistosoma mansoni]	256072954	109 kDa	99%
hypothetical protein [Schistosoma mansoni]	256083090	530 kDa	99%
hypothetical protein [Schistosoma mansoni]	256052785	190 kDa	95%
cleavage and polyadenylation specificity factor [Schistosoma mansoni]	256077072 (+1)	119 kDa	95%
jumonji domain containing protein-related including hairless [Schistosoma mansoni]	256084415	177 kDa	98%
Calcium-binding protein 2 (CaBP2) [Schistosoma mansoni]	256075345	14 kDa	95%
SJCHGC04200 protein [Schistosoma japonicum]	76155947	28 kDa	98%
hypothetical protein [Schistosoma mansoni]	256085531	224 kDa	95%
rab3 interacting molecule (rim)-related [Schistosoma mansoni]	256082383	147 kDa	95%
hypothetical protein [Schistosoma mansoni]	256074206	144 kDa	95%
tyrosine kinase [Schistosoma mansoni]	17907114 (+1)	140 kDa	95%
hypothetical protein [Schistosoma mansoni]	256087905	650 kDa	100%
hypothetical protein [Schistosoma mansoni]	256091720	6 kDa	95%
synaptic ras gtpase activating protein syngap [Schistosoma mansoni]	256082329	171 kDa	95%
protein kinase [Schistosoma mansoni]	256078631	85 kDa	95%
hypothetical protein [Schistosoma mansoni]	256092840	152 kDa	95%
50S ribosomal protein L1 [Schistosoma mansoni]	256070669	46 kDa	95%
hypothetical protein [Schistosoma mansoni]	256079344	655 kDa	98%
fuse-binding protein-interacting repressor siahbp1 [Schistosoma mansoni]	256073105	58 kDa	95%
SJCHGC03045 protein [Schistosoma japonicum]	56759484	18 kDa	95%
calcyphosine/tpp [Schistosoma mansoni]	256074196	24 kDa	95%

heparan sulfate n-deacetylase/n-sulfotransferase [Schistosoma mansoni]	256074757	113 kDa	95%
hypothetical protein [Schistosoma mansoni]	256089269 (+1)	189 kDa	95%
SUMO ligase [Schistosoma mansoni]	256088034	127 kDa	95%
unknown [Schistosoma japonicum]	56757370 (+1)	19 kDa	95%
unknown [Schistosoma japonicum]	189502808	10 kDa	95%
dna2/nam7 helicase family member [Schistosoma mansoni]	256071194	185 kDa	98%
SJCHGC00905 protein [Schistosoma japonicum]	56757477	40 kDa	95%
hypothetical protein [Schistosoma mansoni]	256082836	61 kDa	95%
hypothetical protein [Schistosoma mansoni]	256052614	235 kDa	95%
hypothetical protein [Schistosoma mansoni]	256075438	6 kDa	95%
hypothetical protein [Schistosoma mansoni]	256078687	121 kDa	95%
SJCHGC05123 protein [Schistosoma japonicum]	76152647	13 kDa	95%
hypothetical protein [Schistosoma mansoni]	256082013	164 kDa	95%
SJCHGC08043 protein [Schistosoma japonicum]	56757251	14 kDa	95%
hypothetical protein [Schistosoma mansoni]	256071779	102 kDa	95%
zinc finger protein [Schistosoma mansoni]	256073423 (+1)	176 kDa	95%
receptor protein tyrosine phosphatase r (pcptp1) [Schistosoma mansoni]	256083417	109 kDa	95%
SJCHGC02359 protein [Schistosoma japonicum]	76155807	27 kDa	95%
SJCHGC09453 protein [Schistosoma japonicum]	56758882 (+1)	89 kDa	95%
SJCHGC07328 protein [Schistosoma japonicum]	76155995	30 kDa	95%
rabphilin-3a [Schistosoma mansoni]	256078602	158 kDa	95%
o-linked n-acetylglucosamine transferase ogt [Schistosoma mansoni]	256079484	119 kDa	95%
neuroglian; septate junction protein [Schistosoma mansoni]	256079750	89 kDa	95%
hypothetical protein [Schistosoma mansoni]	256076534	103 kDa	95%
protein tyrosine phosphatase [Schistosoma mansoni]	256087207	182 kDa	95%
SJCHGC09129 protein [Schistosoma japonicum]	56753359	62 kDa	95%
SPFH domain / Band 7 family [Schistosoma mansoni]	256084861	61 kDa	95%
glycerol 3-phosphate dehydrogenase [Schistosoma mansoni]	55793504 (+1)	38 kDa	95%
hypothetical protein [Schistosoma mansoni]	256081613	164 kDa	95%
SJCHGC03335 protein [Schistosoma japonicum]	76152722	14 kDa	95%
subfamily C1A unassigned peptidase (C01 family) [Schistosoma mansoni]	256082975 (+1)	168 kDa	95%
SJCHGC04015 protein [Schistosoma japonicum]	76153535	30 kDa	95%
hypothetical protein [Schistosoma mansoni]	256083856	199 kDa	95%
SJCHGC09644 protein [Schistosoma japonicum]	56752645	13 kDa	95%
butyrate induced transcript [Schistosoma mansoni]	256070201	51 kDa	95%
SJCHGC05098 protein [Schistosoma japonicum]	76154961	41 kDa	95%
SJCHGC06021 protein [Schistosoma japonicum]	56754708	38 kDa	95%
AF310263_1 heat shock protein HSP60 [Schistosoma mansoni]	21634531	58 kDa	95%
SJCHGC01869 protein [Schistosoma japonicum]	56758970 (+1)	104 kDa	95%
hypothetical protein [Schistosoma mansoni]	256053178	44 kDa	95%
patched 1 [Schistosoma mansoni]	256073803	189 kDa	95%
SJCHGC08921 protein [Schistosoma japonicum]	76152891	25 kDa	95%
chondroitin sulfate proteoglycan [Schistosoma mansoni]	256075737 (+1)	144 kDa	95%
hypothetical protein [Schistosoma mansoni]	256092679	5 kDa	95%
zinc finger protein [Schistosoma mansoni]	256085594	41 kDa	95%

rgpr-related [Schistosoma mansoni]	256070647	134 kDa	95%
hypothetical protein [Schistosoma mansoni]	256052579	114 kDa	95%
SJCHGC08599 protein [Schistosoma japonicum]	76156331	21 kDa	95%
hypothetical protein [Schistosoma mansoni]	256079549	29 kDa	95%
rhoptry protein [Schistosoma mansoni]	256089034	131 kDa	95%
protein phosphatase-2b [Schistosoma mansoni]	256071826	67 kDa	95%
unknown [Schistosoma japonicum]	189503038 (+1)	40 kDa	95%
protein kinase [Schistosoma mansoni]	256084995 (+1)	161 kDa	95%
protein phosphatase 2C [Schistosoma mansoni]	256079476	43 kDa	95%
hypothetical protein [Schistosoma mansoni]	256075283 (+1)	19 kDa	95%
hypothetical protein [Schistosoma mansoni]	256077076	41 kDa	95%
tubulin tyrosine ligase [Schistosoma mansoni]	256083385	156 kDa	95%
sec24-related A B [Schistosoma mansoni]	256072734	65 kDa	95%
SJCHGC04300 protein [Schistosoma japonicum]	76156931	19 kDa	95%
U2 small nuclear ribonucleoprotein A [Schistosoma mansoni]	256089883	8 kDa	95%
hypothetical protein [Schistosoma mansoni]	256087057	31 kDa	95%
SJCHGC04323 protein [Schistosoma japonicum]	76154785	27 kDa	95%
hypothetical protein [Schistosoma japonicum]	29841344 (+1)	35 kDa	95%
hypothetical protein [Schistosoma mansoni]	256088720	150 kDa	98%
tubulin tyrosine ligase [Schistosoma mansoni]	256084896	124 kDa	98%
SJCHGC08047 protein [Schistosoma japonicum]	56755159	22 kDa	95%
hypothetical protein [Schistosoma mansoni]	256085614	241 kDa	95%
hypothetical protein [Schistosoma mansoni]	256089955	15 kDa	95%
SJCHGC00707 protein [Schistosoma japonicum]	56757317	49 kDa	95%
family C19 unassigned peptidase (C19 family) [Schistosoma mansoni]	256074515	100 kDa	95%
SJCHGC03722 protein [Schistosoma japonicum]	56756527	22 kDa	95%
hypothetical protein [Schistosoma mansoni]	256067062	19 kDa	95%
hypothetical protein [Schistosoma mansoni]	256076953	32 kDa	95%
Membrane-bound O-acyltransferase domain-containing protein 5 [Schistosoma japonicum]	257215702 (+1)	18 kDa	95%
ras GTP exchange factor [Schistosoma mansoni]	256072383	234 kDa	95%
hypothetical protein [Schistosoma mansoni]	256076913	30 kDa	95%
phosphoglucomutase [Schistosoma mansoni]	256086891	65 kDa	95%
hypothetical protein putative DEAD/DEAH box helicase, with helicase C-terminal domain [S. j]	29840997 (+3)	40 kDa	95%
hypothetical protein [Schistosoma mansoni]	256086242	191 kDa	95%
ADP-ribosylation factor arf [Schistosoma mansoni]	256087483	21 kDa	95%
SJCHGC06036 protein [Schistosoma japonicum]	76154626	29 kDa	95%
hypothetical protein [Schistosoma mansoni]	256092191	18 kDa	95%
protein arginine n-methyltransferase [Schistosoma mansoni]	256072449	179 kDa	95%
SJCHGC04826 protein [Schistosoma japonicum]	76153968	25 kDa	95%
tegumental protein [Schistosoma mansoni]	256052720	21 kDa	95%
lamin [Schistosoma mansoni]	256083880	240 kDa	95%
hypothetical protein [Schistosoma mansoni]	256085438	29 kDa	95%
tyrosine kinase [Schistosoma mansoni]	256071295	96 kDa	95%
pangolin [Schistosoma mansoni]	256071577	110 kDa	95%
dynein heavy chain [Schistosoma mansoni]	256052216	445 kDa	95%
hypothetical protein [Schistosoma mansoni]	256074985	80 kDa	95%

DNA-directed rna polymerase II subunit [Schistosoma mansoni]	256073421	149 kDa	95%
fatty acid binding protein [Schistosoma mansoni]	256079454 (+1)	15 kDa	95%
nitrilase-related [Schistosoma mansoni]	256081450 (+1)	61 kDa	95%
SJCHGC04342 protein [Schistosoma japonicum]	76156998	17 kDa	95%
rap1 and [Schistosoma mansoni]	256084184 (+3)	21 kDa	99%
zinc finger protein [Schistosoma mansoni]	256087171	152 kDa	98%
SJCHGC09807 protein [Schistosoma japonicum]	60701027	10 kDa	95%
DEAD box ATP-dependent RNA helicase [Schistosoma mansoni]	256082050	69 kDa	95%
proteasome subunit alpha type 5 [Schistosoma japonicum]	222709117 (+4)	27 kDa	95%
hypothetical protein [Schistosoma mansoni]	256062392	56 kDa	95%
transcription intermediary factor 1-related [Schistosoma mansoni]	256086332	78 kDa	95%
hypothetical protein [Schistosoma mansoni]	256081312	29 kDa	95%
protoheme IX farnesyltransferase [Schistosoma mansoni]	256081642	67 kDa	95%
tyrosine kinase [Schistosoma mansoni]	256072750 (+1)	119 kDa	95%
similar to XM_080170 misexpression suppressor of KSR 2 in Drosophila melanogaster [Schistosoma japonicum]	29841008 (+3)	47 kDa	95%
type 2 dopamine receptor [Schistosoma mansoni]	238886051	78 kDa	95%
hypothetical protein [Schistosoma mansoni]	256072157	21 kDa	95%
similar to GenBank Accession Number BC009478 leucine zipper protein, RP42 homologin Homo sapiens [S. j]	29841106 (+3)	31 kDa	95%
SJCHGC07242 protein [Schistosoma japonicum]	56753099	15 kDa	95%
SJCHGC06913 protein [Schistosoma japonicum]	76152927	25 kDa	95%
SJCHGC00996 protein [Schistosoma japonicum]	171473843	27 kDa	95%
hypothetical protein [Schistosoma mansoni]	256073684	23 kDa	95%
titin [Schistosoma mansoni]	256080653	69 kDa	95%
serine/threonine protein kinase [Schistosoma mansoni]	256080865	100 kDa	95%
zinc finger protein [Schistosoma mansoni]	256088314	53 kDa	95%
nicotinic acetylcholine receptor non-alpha subunit precursor [Schistosoma bovis]	40317593	84 kDa	95%
rrp4 [Schistosoma mansoni]	256052273	30 kDa	95%
helicase [Schistosoma mansoni]	256083283 (+1)	166 kDa	95%
SJCHGC01145 protein [Schistosoma japonicum]	56759240	27 kDa	95%
glycosyltransferase-related [Schistosoma mansoni]	256079175	89 kDa	95%
hypothetical protein [Schistosoma mansoni]	256088968	472 kDa	95%
hypothetical protein [Schistosoma mansoni]	256090678	117 kDa	95%
SJCHGC06411 protein [Schistosoma japonicum]	76162933	11 kDa	95%
histone H2A [Schistosoma mansoni]	256073500	26 kDa	95%
ribonuclease z chloroplast [Schistosoma mansoni]	256081658 (+2)	22 kDa	95%
voltage-gated potassium channel [Schistosoma mansoni]	256092890	31 kDa	95%
SJCHGC01540 protein [Schistosoma japonicum]	56757737	14 kDa	95%
SJCHGC08106 protein [Schistosoma japonicum]	76155945	23 kDa	95%
thioredoxin peroxidase 3 [Schistosoma mansoni]	256071152 (+1)	25 kDa	95%
red protein (ik factor) (cytokine ik) [Schistosoma mansoni]	256071154	67 kDa	95%
testis development protein nyd-sp29 [Schistosoma mansoni]	256075433	120 kDa	95%
ankyrin repeat-containing [Schistosoma mansoni]	256079395	31 kDa	95%
ferritin [Schistosoma mansoni]	256079612	21 kDa	95%
hypothetical protein [Schistosoma mansoni]	256080624	116 kDa	95%

serine/threonine protein kinase [Schistosoma mansoni]	256080877	193 kDa	95%
putative ubiquitin specific protease 16 isoform a [Schistosoma japonicum]	257215860	17 kDa	95%
Gap-Pol polyprotein [Schistosoma japonicum]	254587273	147 kDa	95%
40S ribosomal protein S30 [Schistosoma mansoni]	256073239	15 kDa	95%
serine/threonine protein kinase [Schistosoma mansoni]	256084662	93 kDa	95%
hypothetical protein [Schistosoma mansoni]	256084950	51 kDa	95%
hypothetical protein [Schistosoma mansoni]	256085248	10 kDa	95%
mitochondrial chaperone BCS1 [Schistosoma mansoni]	256086434	40 kDa	95%
chromodomain helicase DNA binding protein [Schistosoma mansoni]	256052547	224 kDa	95%
cathepsin F (C01 family) [Schistosoma mansoni]	256077193 (+1)	52 kDa	95%
nuclear pore complex protein nup93 (nucleoporin nup93) (dead eye protein) [S. m]	256090244	106 kDa	95%
22.6 kDa protein [Schistosoma bovis]	186462273 (+3)	23 kDa	95%
actin [Schistosoma bovis]	186462275 (+1)	42 kDa	95%
protocatechuate dioxygenase [Schistosoma mansoni]	256050873	25 kDa	95%
cdc6 [Schistosoma mansoni]	256052129	86 kDa	95%
elongation factor 1-alpha (ef-1-alpha) [Schistosoma mansoni]	256054627 (+6)	24 kDa	95%
hypothetical protein [Schistosoma mansoni]	256078540	64 kDa	95%
zinc finger protein [Schistosoma mansoni]	256079061 (+1)	50 kDa	95%
MHC class I antigen [Schistosoma japonicum]	282892228 (+3)	11 kDa	95%
hypothetical protein [Schistosoma mansoni]	256081664	12 kDa	95%
hypothetical protein [Schistosoma mansoni]	256077048	16 kDa	95%
glutamate receptor NMDA [Schistosoma mansoni]	256079501	86 kDa	95%
E1b-55kD-associated protein [Schistosoma mansoni]	256082626	116 kDa	95%
hypothetical protein [Schistosoma mansoni]	256087867	358 kDa	95%
SJCHGC05323 protein [Schistosoma japonicum]	76154204	32 kDa	95%

Supplemental table 2

Identified Proteins (105)	Accession Number	Molecular Weight
AF418550_1 albumin precursor [Schistosoma mansoni]	15808978	68 kDa
family S9 non-peptidase homologue (S09 family) [Schistosoma mansoni]	256077324	126 kDa
nAChR subunit [Schistosoma mansoni]	256077731 (+1)	70 kDa
receptor tyrosine phosphatase type r2a [Schistosoma mansoni]	256078184	267 kDa
hypothetical protein [Schistosoma mansoni]	256082699	38 kDa
hypothetical protein [Schistosoma mansoni]	256077326	117 kDa
SJCHGC09815 protein [Schistosoma japonicum]	60701195	10 kDa
ryanodine receptor related [Schistosoma mansoni]	256085300	559 kDa
SJCHGC05123 protein [Schistosoma japonicum]	76152647	13 kDa
actin [Schistosoma mansoni]	256079405 (+1)	42 kDa
SJCHGC00905 protein [Schistosoma japonicum]	56757477	40 kDa
neuroglian; septate junction protein [Schistosoma mansoni]	256079750	89 kDa

subfamily C1A unassigned peptidase (C01 family) [Schistosoma mansoni]	256082975 (+1)	168 kDa
elongation factor ts [Schistosoma mansoni]	256079923	17 kDa
hypothetical protein [Schistosoma mansoni]	256087454	328 kDa
SJCHGC04200 protein [Schistosoma japonicum]	76155947	28 kDa
microtubule associated protein xmap215 [Schistosoma mansoni]	256081011 (+1)	227 kDa
SJCHGC09129 protein [Schistosoma japonicum]	56753359	62 kDa
cell division control protein [Schistosoma mansoni]	256072954	109 kDa
hypothetical protein [Schistosoma mansoni]	256083856	199 kDa
calcyphosine/tpp [Schistosoma mansoni]	256074196	24 kDa
hypothetical protein [Schistosoma mansoni]	256085531	224 kDa
family C48 unassigned peptidase (C48 family) [Schistosoma mansoni]	256076943 (+1)	65 kDa
hypothetical protein [Schistosoma mansoni]	256083090	530 kDa
dynein heavy chain [Schistosoma mansoni]	256052216	445 kDa
hypothetical protein [Schistosoma mansoni]	256087905	650 kDa
SJCHGC08043 protein [Schistosoma japonicum]	56757251	14 kDa
SJCHGC03335 protein [Schistosoma japonicum]	76152722	14 kDa
unknown [Schistosoma japonicum]	56757370 (+1)	19 kDa
hypothetical protein [Schistosoma mansoni]	256086641	66 kDa
family C19 unassigned peptidase (C19 family) [Schistosoma mansoni]	256074515	100 kDa
SJCHGC02166 protein [Schistosoma japonicum]	76155164	15 kDa
small nuclear ribonucleoprotein [Schistosoma mansoni]	256088837 (+2)	111 kDa
hypothetical protein [Schistosoma mansoni]	256053129	79 kDa
SJCHGC09167 protein [Schistosoma japonicum]	76155403	41 kDa
short chain dehydrogenase [Schistosoma mansoni]	256087970	72 kDa
SJCHGC09592 protein [Schistosoma japonicum]	56758766	27 kDa
ras GTP exchange factor [Schistosoma mansoni]	256072383	234 kDa
hypothetical protein [Schistosoma mansoni]	256074206	144 kDa
DEAD (Asp-Glu-Ala-Asp) box polypeptide 56 [Schistosoma japonicum]	257215840 (+2)	60 kDa
zinc finger protein [Schistosoma mansoni]	256073423 (+1)	176 kDa
dynein heavy chain [Schistosoma mansoni]	256083867	435 kDa
chondroitin sulfate proteoglycan [Schistosoma mansoni]	256075737	144 kDa
SJCHGC03650 protein [Schistosoma japonicum]	76152950	15 kDa
DEAD box ATP-dependent RNA helicase [Schistosoma mansoni]	256083681 (+1)	104 kDa
protein phosphatase 2C [Schistosoma mansoni]	256079476 (+1)	43 kDa
hypothetical protein [Schistosoma mansoni]	256070279 (+1)	37 kDa
SJCHGC00996 protein [Schistosoma japonicum]	171473843	27 kDa
hypothetical protein [Schistosoma mansoni]	256072819	35 kDa
SJCHGC05701 protein [Schistosoma japonicum]	76156121	15 kDa
SJCHGC07378 protein [Schistosoma japonicum]	76153885	19 kDa
ribonuclease z chloroplast [Schistosoma mansoni]	256081658 (+2)	22 kDa
DEAD box ATP-dependent RNA helicase [Schistosoma mansoni]	256082050	69 kDa
hypothetical protein [Schistosoma japonicum]	29841344 (+1)	35 kDa
hypothetical protein [Schistosoma mansoni]	256074299	36 kDa
neurotracting/lsamp/neurotrimin/obcam related cell adhesion molecule [Schistosoma mansoni]	256070234	138 kDa
hypothetical protein [Schistosoma mansoni]	256078536	79 kDa
hypothetical protein [Schistosoma mansoni]	256081534	229 kDa
hypothetical protein [Schistosoma mansoni]	256073809	29 kDa
[ACP] S-malonyltransferase [Schistosoma mansoni]	256084354	42 kDa

nalp (nacht leucine rich repeat and pyrin domain containing)-related [Schistosoma mansoni]	256075415	76 kDa
aminopeptidase P1 (M24 family) [Schistosoma mansoni]	256080084	59 kDa
AF310263_1 heat shock protein HSP60 [Schistosoma mansoni]	21634531	58 kDa
tegumental protein [Schistosoma mansoni]	256084811	21 kDa
unknown [Schistosoma japonicum]	56755549 (+1)	13 kDa
SJCHGC05138 protein [Schistosoma japonicum]	76162618	28 kDa
hypothetical protein [Schistosoma mansoni]	256076092	36 kDa
SJCHGC06082 protein [Schistosoma japonicum]	29841130	12 kDa
SJCHGC07328 protein [Schistosoma japonicum]	76155995	30 kDa
hypothetical protein [Schistosoma mansoni]	256079092	42 kDa
protein phsophatase-2a [Schistosoma mansoni]	256076428	35 kDa
SJCHGC09147 protein [Schistosoma japonicum]	76152945	27 kDa
hypothetical protein [Schistosoma mansoni]	256071130	32 kDa
peter pan-related [Schistosoma mansoni]	256066569	164 kDa
brefeldin A-inhibited guanine nucleotide-exchange protein [Schistosoma mansoni]	256075113	228 kDa
DNA polymerase theta [Schistosoma mansoni]	256074218	103 kDa
dna2/nam7 helicase family member [Schistosoma mansoni]	256071194	185 kDa
family M13 unassigned peptidase (M13 family) [Schistosoma mansoni]	256087512 (+1)	41 kDa
receptor-type adenylate cyclase [Schistosoma mansoni]	256062386	131 kDa
lupus la ribonucleoprotein [Schistosoma mansoni]	256074055	51 kDa
ribonuclease III [Schistosoma mansoni]	256077012	102 kDa
hypothetical protein [Schistosoma mansoni]	256089269	189 kDa
PI3kinase [Schistosoma mansoni]	256087251	91 kDa
hypothetical protein [Schistosoma mansoni]	256071250	127 kDa
CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase [Schistosoma mansoni]	256087890	55 kDa
SJCHGC04015 protein [Schistosoma japonicum]	76153535	30 kDa
myosin XV [Schistosoma mansoni]	256072292	280 kDa
malic enzyme [Schistosoma mansoni]	256074831 (+1)	60 kDa
hypothetical protein [Schistosoma mansoni]	256075042	169 kDa
sec15 [Schistosoma mansoni]	256078962	91 kDa
cdc37-related [Schistosoma mansoni]	256083550	45 kDa
ras GTP exchange factor son of sevenless [Schistosoma mansoni]	256092898 (+1)	178 kDa
sec24-related C d [Schistosoma mansoni]	256082903	126 kDa
voltage-gated potassium channel [Schistosoma mansoni]	256092890	31 kDa
SJCHGC09466 protein [Schistosoma japonicum]	76154754	26 kDa
lysosomal alpha-mannosidase (mannosidase alpha class 2b member 1) [Schistosoma mansoni]	256093034	89 kDa
hypothetical protein [Schistosoma mansoni]	256071051	36 kDa
taspase-1 (T02 family) [Schistosoma mansoni]	256072506	40 kDa
FOG precursor [Schistosoma mansoni]	256088771	69 kDa
hypothetical protein [Schistosoma mansoni]	256075373	37 kDa
A Chain A, Crystal Structure Of The Wild Type Thioredoxin Glutatione Reductase From Schistosoma Mansoni In Complex With Auranofin	256599739	65 kDa
hypothetical protein [Schistosoma mansoni]	256072322	141 kDa
unknown [Schistosoma japonicum]	189502920 (+1)	45 kDa
protocatechuate dioxygenase [Schistosoma mansoni]	256050873	25 kDa
homeodomain transcription factor (phtf) [Schistosoma mansoni]	256088476	147 kDa

McGill

February 18, 2014

Animal Certificate

This is to certify that Dr. Momar Ndao, Department of Medicine, Montreal General Hospital (RI-MUHC), currently holds an approved Animal Use Protocol # 2010-5807 with McGill University and its Affiliated Hospitals' Research Institutes for the following project:

Animal Use Protocol Title: Identification of biomarkers for the development of new vaccines against Schistosomiasis using proteomic technology

Start date: February 1, 2014

Expiration date: February 1, 2015

McGill University and Affiliated Hospitals Research Institutes recognize the importance of animal research in our efforts to further our knowledge of natural processes, diseases and conservation. Research, educational and testing projects are conducted with full commitment to the wellbeing of the animal subjects. In order to limit animal use to meritorious research or educational projects, the institution relies on stringent peer review processes, along with assessment of ethical issues by the Animal Care Committee. McGill University recognizes that the use of animals in research, teaching and testing carries significant responsibilities. The institution will continue to develop and maintain guidelines and regulations, following the high standards established by the Canadian Council on Animal Care. It is committed to conducting the highest-quality research and to providing animals with the best care.

Claude Lalande
Assistant Director, Animal Compliance Office
Office of Vice-Principal (Research and International Relations)
Room 429, James Administration Building, McGill University
845 Sherbrooke Street West, Montreal, Quebec, Canada H3A 0G4