

***Schistosoma mansoni* and
Host-Parasite Interactions**

Saskia de Walick

ISBN 978-94-6169-659-5

© Saskia de Walick, 2015

Cover: Magali de Walick

Printed by: Optima Grafische Communicatie, Rotterdam, The Netherlands

***Schistosoma mansoni* and Host-Parasite Interactions**

Schistosoma mansoni en
gastheer-parasiet interacties

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
vrijdag 22 mei 2015 om 9.30 uur

door

Saskia Marie-Claude Annick de Walick

geboren te Voorburg

Promotiecommissie

Promotoren: Prof.dr. A.G.M. Tielens
Prof.dr. H.A. Verbrugh

Overige leden: Prof.dr. M. Yazdanbakhsh
Prof.dr. Ph.G. de Groot
Prof.dr. E.C.M. van Gorp

Copromotor: Dr. J.J. van Hellemond

Ce n'est pas important la guerre des moutons et des fleurs ?
(Is the war between sheep and flowers not important?)

Le Petit Prince
Antoine de Saint-Exupéry

Contents

Chapter 1	General introduction	1
Chapter 2	The proteome of the insoluble <i>Schistosoma mansoni</i> eggshell skeleton	15
Chapter 3	<i>Schistosoma mansoni</i> : The egg, biosynthesis of the shell and interaction with the host	37
Chapter 4	Binding of von Willebrand factor and plasma proteins to the eggshell of <i>Schistosoma mansoni</i>	51
Chapter 5	Evaluation of molecular and serological methods for diagnosing two cases of neuroschistosomiasis	67
Chapter 6	The tegumental surface membranes of <i>Schistosoma mansoni</i> are enriched in parasite-specific phospholipid species	81
Chapter 7	Summarizing discussion	103
	References	117
	Samenvatting	131
Appendix A	Acknowledgements	137
Appendix B	Curriculum vitae	141
Appendix C	List of publications	143
Appendix D	Portfolio	145

Chapter 1

General introduction

1.1 Schistosomiasis

Blood-dwelling parasitic trematodes (flatworms) of the genus *Schistosoma* cause the disease schistosomiasis or Bilharzia. There are 5 different *Schistosoma* species that infect humans and many other infecting different mammals. Over 200 million people worldwide are infected with schistosomes, mainly of the species *S. haematobium*, *S. mansoni* and *S. japonicum* [37, 197]. The disease is endemic in tropical areas and endemicity is dependent on the presence of the intermediate host, an aquatic snail, in fresh surface water. There is a strong variation in epidemiology between regions and localities, depending on local determinants such as irrigation or draining canals and human sanitary conditions [74].

The life cycle of *S. mansoni*

The life cycle of *S. mansoni* is depicted in Fig. 1.1. Infection of the human host occurs upon water contact, where larvae called cercariae are released from the snails and swim in the water in search for their mammalian host. They penetrate the skin. At this point, cercariae lose their tail and the cercarial body transforms into a migrating juvenile worm, a schistosomulum. In the circulation, schistosomula migrate to the lungs. In the case of *S. mansoni* schistosomula further migrate to the portal vein in the liver where male and female worms pair and mature before homing at their final destination, the mesenteric veins surrounding the gut. This occurs about six weeks after skin penetration [74, 155].

In the mesenteric veins, adult worm pairs can reside for many years [37], feeding on blood and meanwhile producing 300 eggs per day [32]. About half of the deposited eggs flow with the circulation and get trapped in the liver, where granulomas are formed around the eggs due to a strong immune response. The other eggs extravasate the vessel wall and pass through the intestinal wall in order to be secreted with the feces from about one week after being released by the female worm. It may take up to six weeks for the eggs to exit the host. However, eggs die within two weeks after oviposition [37], thus not all excreted eggs are viable.

Viable eggs that are released in water hatch and the larvae called miracidia can subsequently infect the intermediate host, the aquatic snail *Biomphalaria glabrata* or *B. pfeifferi*. Within the snail, massive clonal replication of the parasite, now sporocysts, occurs. About 5 weeks after infection, snails start to shed cercariae. Some of the cercariae will infect a human host.

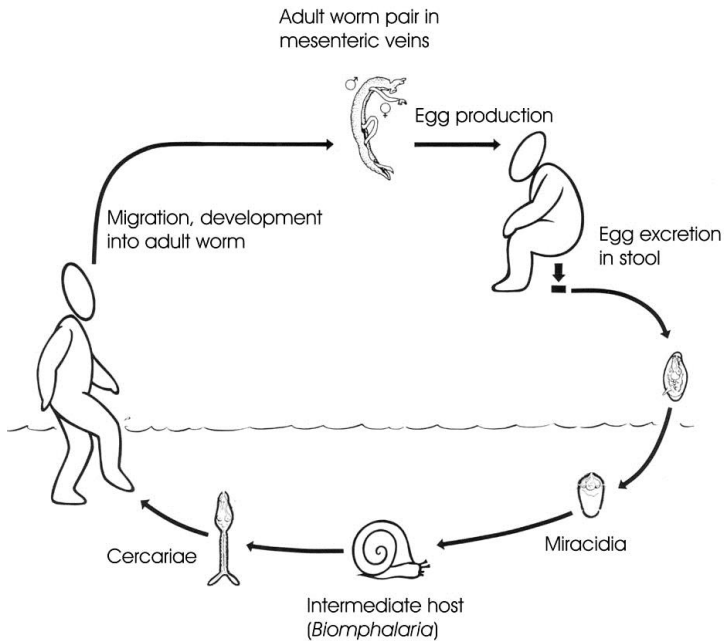


Figure 1.1: The life cycle of *S. mansoni*. (With courtesy of Dr. A.M. Polderman)

Disease symptoms

Infections with *S. mansoni* often occur without notice. However, symptoms may be present in all stages of the infections [75].

The penetration of the skin can cause some local irritation called swimmer's itch. This usually does not last longer than a few hours. When seen, this is most often in primary infections, mainly in migrants and tourists. It is more often seen after infections with non-permissive schistosome species, such as *Trichobilharzia* [35, 75].

Acute schistosomiasis, also called Katayama fever, can occur in primary infections with high numbers of migrating schistosomula [35]. It results from a hypersensitivity T helper 1 T-cell (Th1) response against migrating juvenile worms. The symptoms are flu-like and may include fever, fatigue, myalgia and dry cough. At this stage, antibodies against schistosomes may be found, but stool and urine samples are negative for schistosome eggs. There is a marked eosinophilia and patchy infiltrates can be seen on chest radiography. Usually, symptoms disappear within a few weeks [35, 75].

Abdominal symptoms caused by the migration and positioning of mature worms can occur at a later stage, when worms have matured and migrate to their final destination [74].

Schistosomiasis is most often apparent as a chronic disease. At this stage, much damage can be done of which some is irreversible. The major symptoms of disease are caused by the eggs that elicit a strong immune response. The severity of disease symptoms is dependent on the intensity and the duration of the infection as well as on host immune factors. In and around the intestine the immune reaction to eggs may lead to micro-ulcerations, pseudopolyps and microscopic bleeding causing abdominal pain, loss of appetite, diarrhea, anemia and malnutrition. The more serious disease symptoms are caused by eggs trapped in the liver. The immune reaction causes granulomas to form around the eggs. But it can also cause damage to the liver, leading to fibrosis of the liver, liver enlargement and portal hypertension. It is referred to as hepatosplenic schistosomiasis when portal hypertension leads to splenomegaly, portocaval shunting and/or external or gastrointestinal varices with the potential of fatal bleeding [74].

Ectopic schistosomiasis can develop in many tissues accidentally reached by worms and/or eggs. In advanced hepatosplenic schistosomiasis, portocaval shunts or portopulmonary anastomoses via the azygos vein may form as a consequence of portal hypertension. As a result, eggs may pass into the arterial circulation and get trapped in the lungs [75]. Granulomas have also been found in the skin, adrenal glands, skeletal muscle, spinal cord and in the brain [27]. The most severe form of ectopic schistosomiasis is neuroschistosomiasis, where eggs are present in the central nervous system (CNS). Cerebral involvement can be asymptomatic in immune patients with chronic hepatosplenic schistosomiasis, when eggs are scattered around the brain. Symptomatic cerebral involvement is more often seen in *S. japonicum* infection than in infections with other schistosome species. It usually starts within weeks after the infection [28] and may present with an acute or subacute onset of headaches, seizures, altered sensorium, motor weakness, focal neurological deficit, visual impairment or nystagmus, speech disturbances, and cerebellar symptoms [27].

Acute transverse myelopathy is most often caused by *S. mansoni* or *S. haematobium*. It is a severe disabling condition and is underdiagnosed in endemic areas [27]. Transverse myelopathy due to schistosomiasis is more common in young adults, teenagers and children of the male sex. This may be explained by the higher exposure of male individuals to infected water during playing and working activi-

ties [63]. The transverse myelopathy develops in an early stage of the infection, just after the start of egg deposition and often in the absence of other systemic symptoms [27]. The eggs are deposited *in situ* after improper migration of adult worms to blood vessels of the central nervous system, usually equal or below T6, particularly at T11-L1 [63]. Inflammatory and cellular immune reactions around the eggs mechanically suppress the spinal cord and cause neurological dysfunction. The main clinical symptoms are lower limb weakness that may be severe enough to prevent walking, lower limb pain, usually irradiating to the lower limbs with symmetrical or asymmetrical distribution, deep tendon reflexes abnormalities (hyper, hypo, and areflexia) and bladder, intestinal and sexual dysfunction. Usually, sensory symptoms and pain precede motor symptoms. The time between the initial symptoms and full neurological picture is often less than two weeks [28, 63].

Diagnosis

Diagnosis of an *S. mansoni* infection is based on the microscopic detection of eggs in stool samples. The eggs of *S. mansoni* are relatively large, measuring around $140 \times 60 \mu\text{m}$, and have a characteristic lateral spine. The severity of an infection can be estimated by determination of the amount of eggs per gram feces using the Kato-Katz method [91], although there is a wide day to day variation in egg excretion [7, 45]. Microscopic detection of eggs in stool samples is a simple, cheap and quick method of detection. However, sensitivity is low for infections with a small number of worms [44]. Eggs can also be detected in material obtained from a rectal biopsy. Although a higher sensitivity can be achieved this way [75], rectal biopsies are not routinely done as this is invasive and costly.

There are dipstick tests that detect circulating cathodic antigen (CCA) or circulating anodic antigen (CAA) with monoclonal antibodies. The sensitivity of these tests is modest, but they have shown useful for screening, follow-up of chemotherapy and re-infection, control programmes and epidemiological studies [75].

More sensitive are the serological tests that detect the presence of anti-schistosome antibodies in blood using ELISA or indirect hemagglutination assays (IHA). Seroconversion usually occurs 4 to 8 weeks after infection [73, 75]. Although more sensitive, a major drawback is that test results may remain positive long after the infection is cleared [75]. These tests are therefore very useful for detection of

S. mansoni infections in travelers and other incidentally exposed individuals, but are much less useful in endemic areas.

Schistosome DNA can be detected in stool samples. With real-time PCR, the amount of DNA and thus the intensity of an infection can be quantified [180]. However, due to costs and technical issues, PCRs are rarely performed routinely in low-income endemic areas.

Additional tests can be performed to determine pathology. Esophageal varices can be visualized by endoscopy or contrast radiography, while ultrasound, laparoscopy and wedge biopsy can reveal granulomas or periportal fibrosis [75]. In ectopic schistosomiasis, imaging techniques such as CT or MRI scans may reveal lesions and edema for example around the spinal cord or in the brain. These findings are unspecific for schistosomiasis, but may be of additional diagnostic value [28, 63].

Treatment and control

Praziquantel (PZQ) is the main drug used to treat schistosomiasis. It is cheap, safe and highly effective against all schistosome species. Unfortunately, eggs and juvenile worms are not affected by PZQ and the drug does not prevent reinfection [50, 75]. A treatment consists of one single oral dose in endemic areas where reinfection is likely. In travellers, two treatments with one month interval are given to eradicate all worms [207]. In addition to the low costs and high effectiveness of PZQ, the side-effects of PZQ are mild and transient [50]. They include nausea, vomiting, malaise and abdominal pain. But in heavy infections, massive worm shifts and antigen release after treatment may cause acute colic with bloody diarrhea [75]. PZQ is effective within a few hours after intake [74]. It causes tetanic contractions of adults worms as a result of a rapid influx of calcium ions, and causes disruption of the tegument, the worm's surface, after which worms are cleared by the immune cells [50].

Although PZQ treatment does not prevent reinfection, it may help to develop increased immunity to reinfection. Adult worms release antigens when they die, inducing a humoral immune reaction that is partially protective against future infections [122]. This indicates that immunity to schistosomes can be acquired [78, 114], a finding which is further supported by the fact that the rate and intensity of infection in endemic areas generally decreases with age, i.e. children are more often and more heavily infected than adults. However, no successful vaccine has

been developed so far [37].

For the treatment of neuroschistosomiasis, it is essential to administer corticosteroids in addition to PZQ [87]. This is because the immune response following PZQ treatment can aggravate the neurological disorder. As corticosteroids are immunosuppressing, they reduce the inflammation around eggs and dying worms, thereby resolving or reducing neurological damage.

1.2 Host-parasite interactions

Immunology

The immune response in schistosomiasis follows a characteristic pattern. In the acute phase, schistosome migrating through tissues induce a Th1 response. This response is characterized by elevated levels of the pro-inflammatory cytokines IFN- γ and interleukin (IL)-2 [76, 135].

However, after about 8 weeks of infection, when worms have matured, have migrated to the mesenteric veins and have started egg production, a Th2 response becomes dominant. This response is characterized by high levels of the cytokines IL-4 and IL-5 and the presence of alternatively activated macrophages, a macrophage phenotype that usually occurs in the context of a Th2 cytokine environment [52]. Furthermore, the Th2 response in schistosomiasis is accompanied by elevated levels of eosinophils and IgE. The elevation of IL-4 and IL-5 is linked to a down-regulation of the Th1 response, which is marked by a decrease in IFN- γ and IL-2 secretion [135]. The switch from Th1 to predominantly Th2 is initiated by the deposition of eggs by mature female worms [76, 135]. IL-5 is needed for the development of eosinophilia but has no effect on the circulating levels of IgE [167]. In contrast, IL-4 does increase IgE levels [136].

When the disease becomes chronic, which is around 12 to 16 weeks after infection, the immune response gets dominated by a regulatory T-cell response causing the immune suppression characteristic to chronic schistosomiasis [53, 137]. The granuloma sizes around the eggs now reduce. The induction of regulatory T-cells seems to be an important way of controlling over-vigorous immune responses during the course of chronic schistosome infection [53]. IL-10, a regulatory cytokine, has an important role in tempering both Th1 and Th2 responses. Together with TGF- β , IL-10 redundantly reduces liver pathology by suppressing proinflammatory cytokine production [80, 84].

Egg secretion products

As mentioned above, the majority of disease symptoms in schistosomiasis is due to cellular immune responses towards eggs trapped in the host tissues [75, 76]. Live mature eggs actively secrete proteins, some of which are heavily glycosylated. It has been shown that these glycans in SEA are responsible for the induction of a Th2 response from SEA [129]. The major egg secretion proteins, the glycoproteins IPSE/alpha-1 and Omega-1 are both strong inducers of IL-4 production and Th2 development [59, 164, 178]. IPSE/alpha-1 triggers basophils to produce IL-4 [164], whereas Omega-1 acts on dendritic cells to induce Th2 polarization [59, 178]. Omega-1 can induce Th2 responses even independently of IL-4 [59].

It is this characteristic Th2 response that induces granuloma formation around eggs. In addition, Th2 responses are involved in the development of naturally acquired resistance to reinfection with schistosomes [137]. A T-cell reaction against eggs is also of major importance for the extravasation and excretion of eggs out of the host [49, 54, 90].

The eggshell

Not only the egg secretion products, but also the eggshell itself is immunogenic. The eggshell is the outer layer of the eggs and hence a direct site of interaction with the immune system. It is a hardened and tanned structure made from proteins cross-linked by quinone tanning, rendering eggshell intractable to protease activity. It is formed in the female reproductive tract, where the fertilized oocyte gets surrounded by vitelline cells that contain eggshell precursor proteins that are released upon contractions of the ootype [175]. Tyrosinase activity causes cross-linking of the precursor eggshell proteins within single proteins as well as between neighbouring proteins. The resulting eggshell is protease resistant and very rigid, while the eggshell is at the same time very porous, enabling passage of egg secretion products.

The tegument

Adult worms are able to prevent an adequate immune response, allowing them to reside within the veins for many years. The outer surface of the schistosomes, which is called the tegument, is believed to play an essential role in this. This structure is unique to blood-dwelling trematodes and contains unique proteins and

lipids [21, 184]. The tegument is composed of two closely apposed lipid bilayers with different properties on a layer of fused cells, the syncytium. The syncytium contains inclusion bodies from which the tegument is formed and maintained. The inclusions move apically and merge with the existing plasma membrane, where they release their content, which is then incorporated into the outer bilayer [82, 83, 113].

The bilayers mainly consist of phospholipids and large amounts of cholesterol. On a molar basis they contain more cholesterol than phospholipids [2]. This large amount of cholesterol together with the high content of sphingomyelin and saturated (ether-linked) phospholipid species in the tegumental membranes results in a tight packing of the tegumental membranes, which renders them more rigid in physical terms.

The most abundant phospholipid classes in the tegumental membranes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [151]. This is also true for most eukaryotic membranes. However, the fatty acid composition of phospholipids in the tegumental membranes of schistosomes is different from that of the membranes of the blood cells of the final host [2]. Among the fatty acids of the tegumental membranes are several unconventional fatty acid species that are absent in this host. As schistosomes do not synthesize fatty acids *de novo* and all their fatty acids originate from the host, schistosomes must be capable of modifying fatty acids. It has been shown that schistosomes can modify fatty acids by chain elongation and by the introduction of desaturations [22, 118].

With its surface-enlarging folds, spines, and pits, the tegument appears as typical digestive-absorptive epithelium [70, 81]. Indeed, the tegument is essential for the absorption of nutrients [5, 25, 39, 153]. In addition, the tegument is needed for the uptake of cholesterol and other lipids [120, 152]. There is a low-density lipoprotein (LDL)-like receptor on the surface of the tegument [158], however, phospholipid or cholesterol uptake from LDL has never been demonstrated.

Since the tegument is the interface between the parasite and its host, it is likely to play a role in the protection of the worm against the defence mechanism of the host [111, 112]. It is possible that binding of LDL is a way to hide the worm from the immune system by coating it with host material and thus shielding its own antigens [205]. Other possible immune evasion properties of the tegument are proteolytic degradation of host defence proteins, rigid membrane biophysical properties and a rapid turnover of the tegumental membrane [1]. A possible mechanical function has been assigned to the typical spines that are present on the surface. They were

proposed to prevent occlusion of the veins as blood can flow through them. In addition, the spines could prevent damage caused by complement.

Despite the fact that the tegument contains immunogenic proteins [17, 184], it does not seem to elicit an immune response that lethally harms the worms. Other blood-dwelling flukes have similar tegumental structures, while it is absent in intestinal worms or free-living worms. This suggests that the tegument consisting of a double membrane is specially adapted for worm survival in the blood circulation of their host [113, 199].

Hemostasis

Hemostasis is the process of stopping bleeds. It consists of both clot formation and wound healing. In the intact circulation, clotting is prevented by secretion of thrombomodulin, nitric oxide (NO) and prostaglandin by intact endothelial cells. In the case of vascular injury, blood loss is prevented by vasoconstriction and formation of a platelet plug followed by coagulation.

Platelet plug formation – primary hemostasis

Primary hemostasis involves the formation of a platelet plug at the site of injury in order to reduce blood loss through the injured blood vessel. It requires platelet activation, adhesion to the vessel wall and release of platelet granule content resulting in platelet aggregation. Platelets, by their receptor glycoprotein (GP) Ia/IIa adhere to collagen exposed through the damaged endothelium. This occurs within a few seconds of the injury. The process is stabilized by von Willebrand factor (VWF) which bridges GPIb and subendothelial collagen. Bound platelets get activated and degranulate. They release adenosine diphosphate and thromboxane A₂, which promote platelet aggregation. In addition, platelets release serotonin and thromboplastin, which induces vasoconstriction and stimulates secondary coagulation respectively. Platelets aggregate using fibrinogen and VWF as connecting proteins.

The structure and the role of VWF in hemostasis

VWF is a 250 kDa protein that forms large multimers. It is made by endothelial cells and by megakaryocytes. In megakaryocytes, VWF is stored in α -granules that are later partitioned into platelets. In endothelial cells, VWF is stored in cytoplasmic granules called Weibel-Palade bodies. VWF is released upon platelet activation

and endothelial damage and in response to stimuli including histamine, thrombin, fibrin, vasopressin, epinephrine, dopamine and nitric oxide (NO) [105, 159].

Each VWF monomer contains a number of specific domains in the following order: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK. The A1 domain binds GPIIb, the only receptor for VWF on non-activated platelets. After binding to VWF, platelets get activated. On activated platelets, the GPIIb/IIIa (integrin α IIb β 3) becomes surface exposed. This enables the platelets to also bind the C1 domain of VWF. The A1 domain further binds heparin and collagen. The A3 domain also binds collagen. Hence VWF can serve as a bridging agent between the platelet plug and the extracellular tissue matrix exposed by the damaged vessel wall [105, 159].

The role of endothelium in hemostasis

Intact endothelium inhibits platelet aggregation and coagulation by release of thrombomodulin, NO and prostaglandin I₂. Upon injury, subendothelial collagen gets exposed to the intravascular lumen and passing platelets bind to the collagen with their GPIa/IIa receptor and get activated. Damaged endothelium secretes VWF, which also binds and activates platelets. Hence, when primary hemostasis is initiated, it triggers secondary hemostasis, which comprises the proteolytic clotting factor cascade and results in fibrin fibers and thrombus formation. Tissue Factor (TF) is another initiator of secondary hemostasis. TF is stored in subendothelial cells and released upon damage of the endothelium. Shear stress is also another activator of endothelial cells and thereby an activator of clot formation [177].

Schistosomes and the hemostatic system

Although schistosomes live in the blood circulation, they rarely give hemostatic complications. This is remarkable for an object sizing 0.5 mm in diameter and 5 mm long (sizes of an adult worm pair) in the mesenteric veins, which have a diameter of 1 to 4 mm. Such an obstruction causes turbulence and shear stress, conditions that normally predispose the development of thrombotic events as a result of endothelial damage, activation of platelets and blood coagulation [103, 177]. Also the attachment of the adults to the vessel wall is expected to activate or damage the endothelium [58, 130]. Furthermore, schistosomes provide a foreign surface to blood, an event that is also commonly associated with platelet activation and thrombus formation. However, platelets do minimally adhere to adult schistosome

or isolated tegument [203]. In contrast, platelets adhere massively to eggshell. The adhered platelets showed extensive spreading [203]. It is likely that the binding of platelets to eggshell is essential for egg extravasation from the circulation.

1.3 Thesis outline/scope

The protein composition of different developmental stages of *S. mansoni* has been subject of investigation of many other researchers [15–17, 30, 40, 77, 110, 184]. So far, detailed information on the protein composition of the eggshell was missing, while this structure is a long lasting site of direct interaction between the parasite and the host immune system. We analyzed the protein composition of the *S. mansoni* eggshell. Due to its close contact with the miracidium and host plasma and due to the rigid structure of the eggshell, several technical challenges needed to be dealt with in order to retrieve a clean and analytic fraction of the *S. mansoni* eggshell. The results are presented in chapter 2. In addition to the proteome, eggshell glycans were analyzed.

Chapter 3 describes the formation and composition of the eggshell with the new insights acquired from the study described in chapter 2. In this chapter, possible consequences of the eggshell structure and composition for its interaction with the host are discussed.

One of the interactions between the eggshell and the host is further investigated in chapter 4. As was previously described, platelets bind to eggshell and get activated [203]. We here analyzed the direct binding of host plasma proteins and in particular of VWF to the eggshell of *S. mansoni*. We demonstrated that multiple plasma proteins bind eggshell and that VWF binds eggshell directly through the A1 domain. This led to our hypothesis that binding of platelets, VWF and other plasma proteins is essential for the adhesion of eggs to the endothelium, which is the first step in extravasation from the circulation.

Chapter 5 reports the use of an immunologic assay to diagnose neuroschistosomiasis. As neuroschistosomiasis can rapidly progress into full paralysis with permanent nerve damage, it is of great importance to rapidly diagnose this condition. The therapy for neuroschistosomiasis with immunosuppressive corticosteroids can be detrimental in case of viral or bacterial causes of transverse myelitis. Therefore, it is important to exclude any other infectious cause of myelopathy and to be certain of the diagnosis neuroschistosomiasis. The described method is based on differential humoral immune responses to eggs and worms in plasma and in cere-

bralspinal fluid (CSF). Several other possible molecular and immunologic methods were also evaluated for their usefulness in the diagnosis of neuroschistosomiasis.

The phospholipid composition of the tegumental membranes were analyzed and compared to whole worm and to mammalian cellular membranes. The results are described in chapter 6. In addition, culture supernatants and blood plasma of infected hamsters were analyzed for schistosome lipid secretion products. These analyses showed that tegument contain tegument-specific phospholipids of which species composition is considerably different from whole worm or host cells. These tegument-specific phospholipids could not be detected in serum of infected hamsters nor in culture supernatant. However, this does not necessarily imply that they are not secreted by schistosomes *in situ* in the host.

Chapter 7 summarises the conclusions of the work described in this thesis and possible implications of the results are discussed.

Chapter 2

The proteome of the insoluble *Schistosoma mansoni* eggshell skeleton

Saskia deWalick¹
Michiel L. Bexkens¹
Bas W.M. van Balkom^{2,3}
Ya-Ping Wu⁴
Cornelis H. Smit⁵
Cornelis H. Hokke⁵
Philip G. de Groot⁴
Albert J.R. Heck^{2,6}
Aloysius G.M. Tielens¹
Jaap J. van Hellemond¹

International Journal for Parasitology 2011, 41:523–532.

¹ Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands

² Department of Biomolecular Mass Spectrometry, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

³ Institute for Veterinary Research, IVW/GSAH, Utrecht, The Netherlands

⁴ Department of Haematology, University Medical Center Utrecht, Utrecht, The Netherlands

⁵ Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands

⁶ Netherlands Proteomics Centre, Utrecht, The Netherlands

Abstract

In schistosomiasis, the majority of symptoms of the disease is caused by the eggs that are trapped in the liver. These eggs elicit an immune reaction that leads to the formation of granulomas. The eggshell, which is a rigid insoluble structure built from cross-linked proteins, is the site of direct interaction between the egg and the immune system. However, the exact protein composition of the insoluble eggshell was previously unknown. To identify the proteins of the eggshell of *Schistosoma mansoni* we performed LC-MS/MS analysis, immunostaining and amino acid analysis on eggshell fragments. For this, eggshell protein skeleton was prepared by thoroughly cleaning eggshells in a four-step stripping procedure of increasing strength including urea and SDS to remove all material that is not covalently linked to the eggshell itself, but is part of the inside of the egg, such as Reynolds' layer, von Lichtenberg's envelope and the miracidium. We identified 45 proteins of which the majority are non-structural proteins and non-specific for eggs, but are house-keeping proteins that are present in large quantities in worms and miracidia. Some of these proteins are known to be immunogenic, such as HSP70, GST and enolase. In addition, a number of schistosome-specific proteins with unknown function and no homology to any known annotated protein were found to be incorporated in the eggshell. Schistosome-specific glycoconjugates were also shown to be present on the eggshell protein skeleton. This study also confirmed that the putative eggshell protein p14 contributes largely to the eggshell. Together, these results give new insights into eggshell composition as well as eggshell formation. Those proteins that are present at the site and time of eggshell formation are incorporated in the cross-linked eggshell and this cross-linking does no longer occur when the miracidium starts secreting proteins.

2.1 Introduction

Schistosomiasis is a tropical disease affecting an estimated 200 million people worldwide, mainly in sub-saharan Africa [75]. The disease is caused by a parasitic flatworm of the *Schistosoma* genus which can reside in the veins of its host for many years. Apparently, the host fails to elicit an adequate immune response to the adult worms. In contrast, the eggs provoke a strong immune response and are the main cause of pathology in schistosomiasis. To complete the life cycle and be excreted with the faeces, the *Schistosoma mansoni* eggs have to extravasate from the vessel and penetrate through the intestinal wall, thereby damaging the tissues they pass. This process is dependent on an immune reaction which is elicited by the eggs [49]. Furthermore, the eggshells appear to be potent inducers of platelet activation, which may facilitate extravasation [203]. Although the eggs have to be excreted with the faeces to propagate the life cycle, many of the eggs do not reach the intestines. Instead, they follow the blood flow and are trapped in the liver, where they are responsible for granuloma formation which can lead to fibrosis and portal hypertension with all its complications in chronic disease. The granulomas are a result of massive inflammation around the eggs, due to the strong immune response that they induce. This immune response is skewed towards a T-helper 2 response, which is characteristic for helminth infections.

The *Schistosoma* eggshell is a porous protein structure with microspines on the outer surface [123]. The proteins that form the eggshell are cross-linked by quinone tanning, making the eggshell a very rigid structure [194]. This quinone tanning is a result of tyrosinase activity, which converts tyrosine residues into *o*-quinones. These *o*-quinones react with nucleophiles such as lysine and histidine, resulting in a series of cross-links within a single protein as well as between distinct proteins. Tyrosinase activity has been shown to be essential in eggshell formation [65].

Although the eggs play an important role in development of the disease, the protein composition of their shell, which is the site of direct interaction between the eggs and the immune system, has not yet been reported, even though female-specific proteins, as determined by mRNA expression or by labeled amino acid uptake, have been postulated to be putative eggshell proteins [14, 33, 88, 144].

Recently, a study was published where several protein fractions of the *Schistosoma* egg were analysed by two-dimensional (2-D) gel electrophoresis and mass spectrometry (MS) [110]. These five different fractions consisted of mature and

immature eggs, miracidia, hatch fluid and egg secreted proteins (ESPs). In all of the fractions except ESP, the list of identified proteins was similar, containing a range of proteins from different functional categories. The ESP fraction appeared to contain just a small and unique subset of the total egg proteome, in strong contrast with a previous study where the list of proteins secreted by the eggs was similar to those from the other fractions [30]. The only fraction of the *S. mansoni* egg of which the proteome has not been characterised is the insoluble shell of the egg, which is the actual site of direct interaction with the host.

A published proteomic analysis of eggshell of the closely related *Schistosoma japonicum* identified 520 proteins. The list of proteins of *S. japonicum* eggshell included a wide range of proteins such as putative eggshell proteins, previously characterised egg proteins, motor proteins, chaperones and enzymes [100]. However, this list is probably too extensive as in these samples the eggshells were not purified from all attached material from the inside of the eggs. Proteins from the structures underlying the eggshell are not cross-linked and may be over-represented in the MS analysis as they are easier to detect and identify than the cross-linked eggshell proteins.

Despite its importance in pathogenicity and host-parasite interactions, the composition of the *S. mansoni* eggshell has not been determined. In this study we performed LC-MS/MS on purified eggshell fragments to identify the proteins of which the shell is composed. The results showed that the *Schistosoma* eggshell is not only made from known putative eggshell proteins, but from a range of proteins, some of which are known to be immunogenic. Identification of the eggshell proteins showed that apparently eggshell formation is completed before maturation of the egg.

2.2 Materials and methods

Isolation of eggs

A Puerto Rican strain of *S. mansoni* was maintained in Golden hamsters for which animal ethics was approved (licence EUR1860-11709). Animal care and maintenance was in accordance with institution and governmental guidelines. Eggs were isolated by overnight digestion of livers from infected hamsters in 500 ml 1.8% NaCl with 5 ml collagenase buffer (6.8 mM NaCl, 0.7 mM KCl, 9.2 mM HEPES, 0.8 mM CaCl₂ and 745 U/ml collagenase Type A1 (Sigma, St Louis, MO, USA) pH 7.8)

[166]. Subsequently, for the isolation of eggs, the digested liver suspension was passed over sieves. Undigested liver material was removed by the first two sieves (425 and 180 μm mesh), after which the eggs were collected on the third (45 μm mesh). The eggs were then washed in Dutch spring water (Bar-le-Duc, Utrecht, The Netherlands) and allowed to hatch for at least 3 h. Eggshells were collected, frozen in liquid nitrogen and crushed in a micro-dismembrator S (Braun Biotech Int., Melsungen, Germany) by shaking at 2000 rpm for 2 min. Finally, the eggshell fragments were layered on a 60% (w/v) sucrose cushion and centrifuged at 650g for 2 min. Subsequently, the eggshell pellets were washed three times with MilliQ water to remove remaining sucrose.

Purification of eggshells

In order to remove all attached cellular material from the eggshells, the eggshell fragments were further purified in four sequential steps. First, the eggshell fragments were incubated in 2 M NaCl in PBS at room temperature for 30 min. Next, the eggshell fragments were incubated in 1% (v/v) Triton X-100 in PBS at room temperature for 30 min followed by incubation in 8 M urea with 0.6% β -mercaptoethanol in PBS at room temperature for 30 min. Finally, the eggshell fragments were incubated in 1% (w/v) SDS at 95 °C for 30 min. Eggshell fragments were washed three times after each of the first three purifications steps and five times after the final step.

After each purification step, eggshell fragments were collected for protein identification by LC-MS/MS after trypsin treatment.

Trypsin digestion

Laemmli buffer was added to the eggshell fragments and proteins were incubated at 37 °C for 30 min before loading on a 12% SDS-PAGE. The samples were run until the loading front reached the stacking/running gel interface. The gel was then fixed in 5% acetic acid 30% methanol and subsequently proteins were stained using GelCode Blue reagent (Pierce, Rockford, IL, USA). After destaining in MilliQ water, a single gel piece surrounding the loaded well (containing all proteins and eggshell fragments) was excised and subjected to in-gel trypsin digestion. For this, the gel piece was cut into small pieces, which were rinsed with MilliQ water and treated as described previously [184].

HPLC and MS

Peptides generated by in-gel digestion were analysed by nanoflow liquid chromatography using an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled on-line to a 7-tesla LTQ-FT mass spectrometer (Thermo Electron, Bremen, Germany). The system was operated in a set-up essentially as previously described [116]. ReproSil-Pur C18-AQ, 3 μm (Dr. Maisch, GmbH, Ammerbuch, Germany) was used as a resin for capillary reversed phase chromatography. Peptides were trapped at 5 $\mu\text{l}/\text{min}$ on a 1 cm column (100- μm internal diameter, packed in-house) and eluted to a 15 cm column (50- μm internal diameter, packed in-house) at 150 nl/min on a 60 min gradient from 0% to 50% acetonitrile in 0.1 M acetic acid. The eluent was sprayed via emitter tips (made in-house) butt-connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in Fourier-transform ion cyclotron resonance (FT-ICR) MS with a resolution of 20,000 at a target value of 2,000,000. The three most intense ions were then isolated for accurate mass measurements by a selected ion monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. These ions were then fragmented in the linear ion trap using collision-induced dissociation at a target value of 15,000.

Database searching

Tandem MS were extracted, charge state deconvoluted and deisotoped by BioWorks version 2.0. All MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 2.2.1). Mascot was set up to search the GeneDB_Smansoni_Proteins.v4.0h, assuming the digestion enzyme trypsin and allowing for two missed cleavages. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 50 parts per million (PPM). Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Oxidation of histidine, methionine and tryptophan was specified in Mascot as a variable modification.

Criteria for protein identification

Scaffold (version 3.0, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as

specified by the Peptide Prophet algorithm [92]. Protein identifications were filtered using a false discovery rate of <5% and were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [124]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to one family to satisfy the principles of parsimony. Proteins that were identified in at least two out of three independent experiments were considered as true hits.

Scanning electron micrography with immunogold nanoparticle labeling

Egg or eggshell fragment samples were fixed in 2% glutaraldehyde (Merck, Germany)/HEPES prior to incubation with primary antibody against mouse heat shock protein 70 (HSP70); control samples were mock treated. Then, a second antibody goat anti-mouse 30 nm IgG-Gold nanoparticle (DGMGL-B001, BioAssay Works, LLC, Ijamsville, MD, USA) was used for recognition of the primary antibody. Samples were fixed for a second time with 3% glutaraldehyde/HEPES and subsequently dehydrated in a 80%, 90% and 100% graded series of ethanol and 1,1,1,3,3,3-Hexamethyldisilazane (MP Biomedicals Inc., Solon, Ohio, USA). The samples were sputter-coated with a thin layer of 6.5 nm platinum (spurt density:21.45) in a sputter coater and viewed in a scanning electron microscope (Philips XL30, Philips, Netherlands) with backscatter electron detector and secondary electron detector, Bias (volt) -216 V, Acc V 5.00 kV, work distance 2.9 and magnification 100,000 ×.

Western blots

Soluble egg antigens (SEA), adult worm antigens (AWA) and approximately 1 µg of schistosome GST and rabbit muscle enolase (Sigma) were run through a 12% SDS-PAGE. SEA and AWA were prepared by homogenisation in PBS followed by centrifugation. Purified protein standards (Fermentas, St. Leon-Rot, Germany) were loaded onto the gel. After separation, proteins were transferred to Polyvinylidene Fluoride (PVDF). Pooled sera from three infected hamsters were used in 1:100 dilutions as the primary antibody. Horseradish peroxidase (HRP) conjugated anti-hamster IgG (Abcam, Cambridge, UK) was used as a secondary antibody. Blots were developed using Enhanced Chemiluminescence (ECL) detection reagents (Pierce) on hyperfilm ECL (Amersham, GE healthcare, Diegem, Belgium) according

to the manufacturers' instructions.

Glycan-specific monoclonal antibodies (mAbs) generated from schistosome-infected or immunized mice were obtained and characterised as previously described. The following mAbs were used to detect eggshell-associated glycans in dot blots: 114-5B1 (IgG1; binds to GalNAc β 1-4(Fuc α 1-2Fuc α 1-3)GlcNAc (LDN-DF) [192]); 128-1E7-C (IgM; binds to Fuc α 1-3GalNAc β 1-4(Fuc α 1-3)GlcNAc (F-LDN-F) and Fuc α 1-3GalNAc β 1-4GlcNAc (F-LDN) [41, 149]); 100-4G11 (IgM; binds to Man3GlcNAc2 [41, 191]); 120-1B10 (IgG1; binds to Circulating Anodic Antigen (CAA) [46]); 291-4D10 (IgM; binds to Gal β 1-4(Fuc α 1-3)GlcNAc (Lewis X) [149, 192]).

Rabbit antibodies against p14 were kindly provided by Prof. Dr. P.T. LoVerde (University of Texas Health Science Center, San Antonio, Texas, USA). Eggshell fragments were incubated with antibodies, washed three times and subsequently incubated with HRP conjugated anti-mouse or anti-rabbit immunoglobulin antibodies (Dako, Glostrup, Denmark). After washing, the eggshell fragments were incubated in ECL detection reagent and dotted on Whatman paper. Chemiluminescent signal was captured on hyperfilm ECL.

Monosaccharide composition analysis

A 35 μ l aliquot of eggshell suspension was dried under a flow of nitrogen in a glass vial with insert. Dried eggshells were dissolved in 50 μ l 4 M trifluoroacetic acid, hydrolysed by incubation at 100 °C for 4 h and subsequently dried under a flow of nitrogen. Fluorescent labeling of reducing monosaccharides generated by hydrolysis was performed following the method of Ruhaak *et al.* (2010)[157] with some slight modifications. Briefly, a solution of 23% acetic acid in DMSO was used to dissolve anthranilic acid (Sigma, Germany) to a concentration of 48 mg/ml and subsequently 2-picoline borane complex (Sigma, Germany) was added to a concentration of 107 mg/ml. The monosaccharides were dissolved in 10 μ l of this labeling mix and reacted at 65 °C for 2 h. Labeled monosaccharides were diluted in 190 μ l 0.6% sodium acetate.

Labeled monosaccharides were separated by reverse phase (RP)-HPLC on a Superspher 100 RP-18 endcapped column (250 \times 4 mm; Grom, Germany). Solvent A consisted of 0.1% butylamine, 0.5% phosphoric acid and 1% tetrahydrofuran. Solvent B consisted of solvent A/acetonitrile 50/50. The following gradient conditions were applied: at time t = 0 min, 8% solvent B; at t = 5 min, 8% solvent B; at t = 30

min, 23% solvent B; at t = 32 min, 100% solvent B; at t = 42 min, 100% solvent B; at t = 43 min, 8% solvent B; at t = 60 min, 8% solvent B. The flow rate was 500 μ l/min. Samples were injected in a 10 \times dilution with 0.6% sodium acetate. Fluorescence was detected at 360/420 nm. Monosaccharides were identified on the basis of their retention time compared with a reference monosaccharide mixture. The relative monosaccharide composition was calculated on the basis of fluorescence.

Amino acid analysis of eggshell fragments

The analysis of amino acid composition of purified eggshell fragments was performed by Ansynth service BV, Roosendaal, the Netherlands. Prior to analysis, the purified eggshell fragments were hydrolysed overnight in 6 M HCl at 110 °C. Analyses were performed using a Biochrom amino acid analyser equipped with a Ninhydrin detection system. The amounts of alanine, arginine, aspartate plus asparagine, glutamate plus glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine were determined.

2.3 Results

Purification of the eggshells

The *Schistosoma* eggshell is a porous protein structure with microspines on the outer surface [123]. The proteins that form the eggshell are cross-linked by quinone tanning, making the eggshell a very rigid structure [194]. By light microscopy, the eggshells collected directly after digestion of livers appeared clean (Fig. 2.1A). However, those were expected to contain attached material from the underlying Reynolds' layer, von Lichtenberg's envelope and Lehman's lacuna [123]. To ensure that all of this attached material would be removed, we purified the fragments of the eggshells in four consecutive steps with reagents of increasing strength. After crushing the eggshells, the eggshell fragments were treated consecutively with (i) 2 M NaCl to remove proteins attached exclusively by ionic strength, (ii) 1% (v/v) Triton X-100 to remove proteins attached by hydrophobic interactions, (iii) 8 M urea containing 0.6% β -mercaptoethanol and (iv) 1% SDS at 95 °C to remove all proteins that were linked non-covalently or by disulfide bonds. As the *Schistosoma* eggshell is a rigid structure of which the proteins are extensively cross-linked by quinone tanning, the eggshells were not dissolved after the final purification

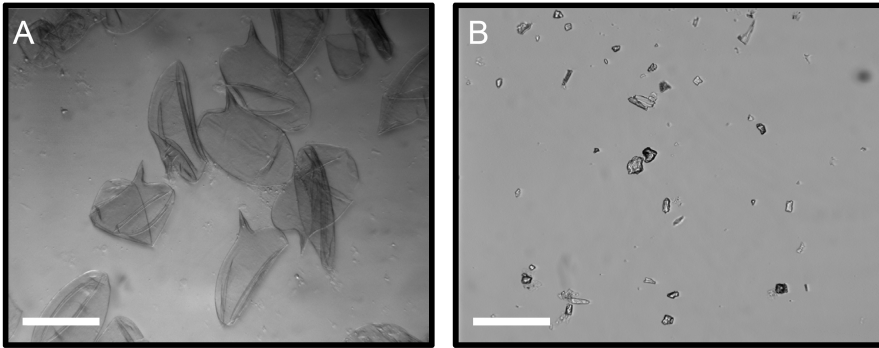


Figure 2.1: Light microscopy of eggshells of *Schistosoma mansoni*. Isolated eggshells before purification (A), eggshell fragments after crushing and purification (B). Bars represent 100 μm and 200 μm in A and B, respectively.

step by treatment with SDS and therefore eggshell fragments were still visible (Fig. 2.1B). We refer to these purified eggshell fragments as the protein skeleton of *S. mansoni* eggshells. Electron scanning microscopy showed that the structure of the eggshell appeared different after each purification step (Fig. 2.2). The eggshell surface directly after isolation is a three-dimensional (3-D) structure with spines and pores similar to previous descriptions [140, 160]. The surface became smooth after NaCl and Triton X-100 treatment of the eggshell fragments. After treatment with 8 M urea, the denatured proteins had swollen. This swelling was reduced after SDS treatment, where the eggshell skeleton was left as a wrinkled and more flattened structure.

Analysis of the eggshell proteome

After each purification step, trypsin digests of the eggshell fragments were used for protein identification by LC-MS/MS. The number of identified proteins declined from 118 directly after eggshell isolation to a final total of 45 proteins in the protein skeleton (Fig. 2.3). The supernatant of the final wash step, after the SDS incubation, did not contain any proteins. The extensive washes after each purification step to remove the liberated proteins thus ensured that all of the identified proteins originated from the eggshell protein skeleton itself and are therefore part of the proteome of the cross-linked eggshell.

The identified eggshell proteins and the number of peptides found in each of

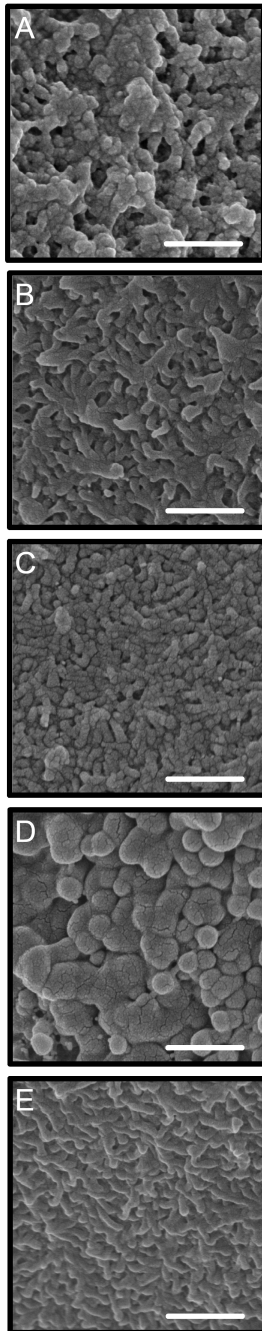


Figure 2.2: Scanning electron microscopy of eggshells of *Schistosoma mansoni*. The surface of eggshell fragments as visualised by scanning electron microscopy directly after crushing (A), after treatment with 2 M NaCl (B), 1% (v/v) Triton X-100 (C), 8 M urea containing 0.6% β -mercaptoethanol (D) and 1% SDS at 95 °C (E). Bars represent 500 nm.

Table 2.1: List of identified proteins in purified eggshell fragments of *Schistosoma mansoni* in three independent experiments. Listed are the number of unique peptides per protein in each of the three experiments. MWs are approximated molecular weights for complete proteins. The percent coverage is the maximum coverage found in the three experiments.

Accession number	Protein name	Protein MW (kDa)	Number of unique peptides			Protein Coverage (%)
			Exp 1	Exp 2	Exp 3	
Energy metabolism						
Smp_002880.1	ATP synthase alpha subunit mitochondrial	60	5	13	2	30
Smp_038100	ATP synthase beta subunit	56	6	9	4	25
Smp_194770	ATP:guanidino kinase (Smc74)	95	2	13	0	21
Smp_024110	Enolase ^a	47	3	5	2	10
Smp_042160.2	Fructose 1,6-bisphosphate aldolase	40	0	4	2	15
Smp_056970.1	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	36	3	9	1	32
Smp_143840	Glycogen phosphorylase	80	2	8	0	14
Smp_038950	Lactate dehydrogenase ^b	36	2	7	1	23
Smp_035270.2	Malate dehydrogenase	31	2	7	0	22
Smp_047370	Malate dehydrogenase	36	2	7	4	23
Smp_130300	Na ⁺ /k ⁺ atpase alpha subunit	93	2	5	1	7
Smp_005880	Phosphoenolpyruvate carboxykinase	70	4	13	2	30
Smp_187370	Phosphoglycerate kinase	18	2	5	1	30
Smp_059790.2	Transketolase	63	2	4	0	9
Protein folding and stress response						
Smp_073880.1	40S ribosomal protein S3A	29	2	4	0	17
Smp_054160	Glutathione S-transferase 28 kDa (GST 28) (GST class-mu)	24	2	4	0	18
Smp_072330.2	Heat shock protein	81	6	25	9	38
Smp_106930.2	Heat shock protein 70	69	17	30	5	46
Smp_069130.2	Heat shock protein 70 (hsp70)-4	94	2	11	1	17
Smp_062420.1	Heat shock protein 70 (hsp70)-interacting protein	31	0	3	2	13
Smp_008545	Heat shock protein HSP60	61	3	10	1	20
Smp_049250	Major egg antigen p40 ^c	40	4	11	4	44
Smp_079770.1	Protein disulfide-isomerase er-60 precursor (erp60)	54	2	4	0	10
Smp_095980	Superoxide dismutase precursor (EC 1.15.1.1)	20	9	3	3	44
Smp_059480	Thioredoxin peroxidase	21	3	4	1	25

Table 2.1 continued

Accession number	Protein name	Protein MW (kDa)	Number of unique peptides			Protein Coverage (%)
			Exp 1	Exp 2	Exp 3	
Cytoskeleton						
Smp_161920	Actin	42	5	16	5	51
Smp_090120.1	Alpha tubulin	50	7	13	2	30
Smp_085540.6	Myosin heavy chain	211	2	9	0	6
Smp_035760	Tubulin beta chain	50	13	23	7	51
Membrane proteins						
Smp_020550	Low-density lipoprotein receptor (ldl)	87	7	9	4	12
Smp_159420	Low-density lipoprotein receptor (ldl)	103	4	4	0	5
Smp_179370	Low-density lipoprotein receptor (ldl)	94	3	3	1	6
Smp_091240.1	Voltage-dependent anion-selective channel	31	2	3	0	12
Protein synthesis						
Smp_099870	Elongation factor 1-alpha (ef-1-alpha)	51	13	14	2	37
Smp_030690	Elongation factor 1-beta	24	2	2	0	11
Smp_143140	Eukaryotic translation elongation factor	22	1	2	2	15
Smp_143150	Eukaryotic translation elongation factor	61	2	7	0	17
Nuclear proteins						
Smp_053290	Histone H4	11	3	8	0	52
Other and unknown functions						
Smp_179260	Alpha-galactosidase/alpha-n-acetylgalactosaminidase	109	5	10	0	13
Smp_174530	Aminopeptidase PILS (M01 family)	111	0	2	2	2
Smp_147890	Rootletin (Ciliary rootlet coiled-coil protein)	234	31	70	8	36
Smp_005860.1	Expressed protein ^d	47	4	7	4	21
Smp_148160	Expressed protein ^e	35	2	4	0	14
Smp_160560	Expressed protein ^f	79	8	8	2	13
Smp_000270	Hypothetical protein ^g	26	2	0	3	21

^a Annotated as phosphopyruvate hydratase.

^b Annotated as malate dehydrogenase.

^c Annotated as heat shock protein.

^d Similar to heterogenous nuclear ribonucleoprotein k.

^e Similar to spermatogenesis-associated protein 6 precursor (SPATA6).

^f Similar to TyrA protein.

^g Similar to FS800, a female-specific protein.

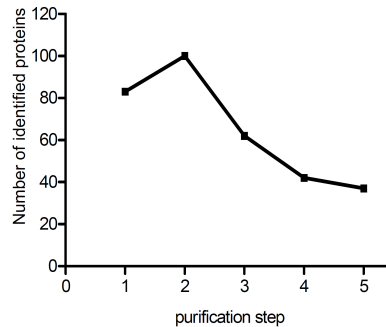


Figure 2.3: The number of proteins identified by MS after each purification step of the eggshell of *Schistosoma mansoni*.

the three independent experiments are listed in Table 2.1. The molecular weights of the identified proteins and the maximum protein coverages are also shown in the table.

Among the identified eggshell proteins, we found some known schistosome antigens, such as major egg antigen p40 and HSP70 [38, 121, 176]. In addition, multiple structural proteins that are normally part of the cytoskeleton of a cell were identified, such as actin and β -tubulin. Extracellular structural proteins, such as fibrin or collagen, were not identified. Furthermore, in this MS analysis we did not identify any of the putative eggshell proteins (see below in Sections *Analysis of the eggshell amino acid composition* and *Discussion* however).

Surprisingly enough, the majority of the identified proteins were non-structural proteins. We identified membrane proteins, cytosolic proteins and nuclear proteins. A collection of enzymes was identified, of which many were glycolytic enzymes (Table 2.1). It is yet unknown whether the enzymes present in the eggshell protein skeleton are still catalytically active *in situ*. However, residual catalytic activity of enzymatic proteins in the eggshell is highly unlikely as these proteins are tightly cross-linked to other proteins and incorporated into the eggshell.

Protein identifications by MS were verified by localising one of the identified eggshell proteins on purified eggshell fragments by immunoscanning electron microscopy. Fig. 2.4 shows HSP70 on the eggshell fragments before and after purification. Its presence validates the protein identifications by MS.

Analysis of eggshell glycoconjugates

Schistosome eggs are known to contain and secrete glycoproteins that carry immunogenic glycans [85]. We investigated whether glycan antigens were still

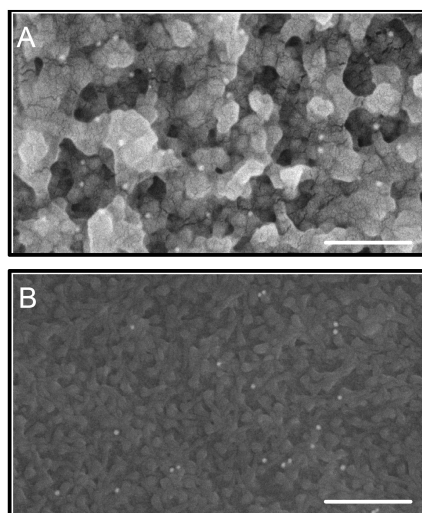


Figure 2.4: Immunoscanning electron microscopy for heat shock protein 70 (HSP70) on eggshell fragments of *Schistosoma mansoni*. HSP70 immunolabeling on unpurified (A) and purified (B) eggshell fragments. Bars represent 500 nm.

present on the eggshell protein skeleton. Blotting of eggshells with a set of schistosome glycan reactive mAbs showed that mAb 128-1E7-C (weak) and 291-4D10 (strong) generate a positive signal, indicating that the glycan epitopes F-LDN(-F) and Lewis X are expressed on eggshell glycans, whereas the other epitopes tested are not expressed or not detectable (Fig. 2.5).

To confirm the occurrence of glycans on eggshells, a monosaccharide composition analysis on hydrolysed eggshells was performed (Table 2.2). The composition analysis indicates the presence of a number of different constituent monosaccharides associated with the eggshells. The occurrence of mannose residues (three in every N-linked glycan core) suggests that N-linked glycans are present, but in addition O-linked glycans or other glycan classes would be in agreement with this overall composition. We have not been able to discriminate between xylose and fucose. In an N-linked glycan from schistosome eggs, up to one xylose residue in each N-glycan may be present. This suggests that at least fucose residues are also present, which is in line with the dot blot results showing staining for fucosylated glycan epitopes. At this stage it is not possible to derive any details about eggshell glycosylation, or which proteins are carrying the putative glycans.

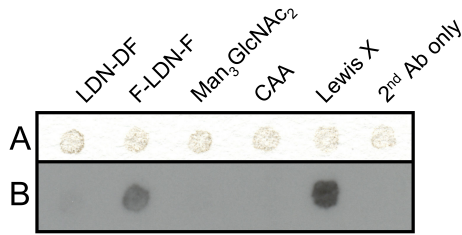


Figure 2.5: Analysis of glycoconjugates on the eggshell of *Schistosoma mansoni*. Dot blots of eggshell protein skeleton were blotted for schistosome-specific glycoconjugates with monoclonal antibodies 114-5B1 (GalNAc β 1-4(Fuc α 1-2Fuc α 1-3)GlcNAc, (LDN-DF)), 128-1E7-C (Fuc α 1-3GalNAc β 1-4(Fuc α 1-3)GlcNAc (F-LDN-F)), 100-4G11 (Man $_3$ GlcNAc $_2$), 120-1B10 (Circulating Anodic Antigen (CAA)), 291-4D10 (Gal β 1-4(Fuc α 1-3)GlcNAc, (Lewis X)) and binding was detected with Horseradish peroxidase (HRP) conjugated anti-hamster IgG (Abcam, Cambridge, UK) as secondary antibody (Ab). The dotted eggshell fragments are shown in panel A, chemiluminescent signal was captured on hyperfilm (panel B).

Table 2.2: Monosaccharide composition analysis of purified eggshell fragments of *Schistosoma mansoni*.

Monosaccharide	Relative ratios (Man set to 3)
Man	3
GlcNAc	10
Gal	3
GalNAc	3
Fuc/Xyl ^a	4
Glc	3

^a Not discriminated due to overlapping peaks.

Analysis of the eggshell amino acid composition

Putative eggshell proteins were identified in the late 1980s by searching for specific cDNAs that are only expressed in mature females and not in males, immature female worms, miracidia or cercariae. Examples of female-specific proteins that fulfill these criteria are p14 [14] and p48 [33]. These putative eggshell proteins appeared to have a characteristic amino acid composition, containing either high glycine and tyrosine levels (p14) or high lysine and tyrosine levels (p48). Although these proteins are expected to be abundantly present in the protein skeleton of the eggshells, we did not identify these proteins in our proteomic analysis.

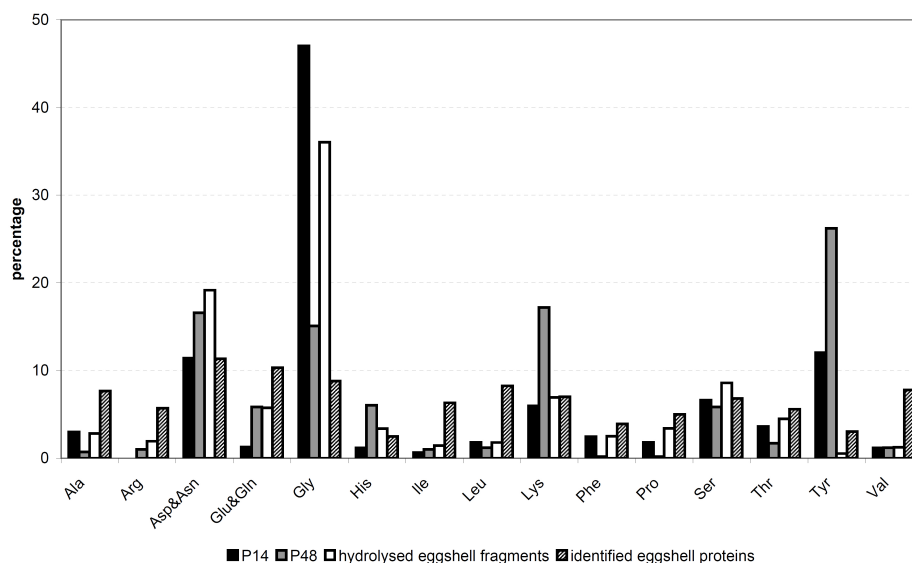


Figure 2.6: Amino acid composition of the purified eggshell skeleton of *Schistosoma mansoni*. The contribution of the various amino acids to the proteins p14 (black Bobek *et al.* (1988)[14]), p48 (gray Chen *et al.* (1992)[33]), hydrolysed eggshell protein skeleton (white, this study) and the average of the eggshell proteins that we identified by MS (dashed bars, listed in Table 2.1).

Since p14 and p48 both contain high amounts of tyrosine residues, which are modified and cross-linked during the process of quinone tanning, identification of these proteins by peptide fingerprinting using MS is hampered by the post-translational modifications. Therefore, we performed amino acid analysis on the purified eggshell fragments (Fig. 2.6) to see whether it is likely that p14 and p48 were present in our sample, and hence are part of the insoluble protein skeleton of the eggshell. The amino acid composition of the purified eggshell skeleton was very similar to previous reports [24, 150, 195]. High glycine levels (36%) suggest that p14 is indeed present in the purified eggshell fragments and is a highly abundant eggshell protein. In female worms, p14 mRNA, which is known as the F10 gene, is the most abundant transcript [128]. Using immunostaining we were indeed able to confirm the presence of p14 in our samples of purified eggshells (Fig. 2.7).

Although p48 was expected to be part of the eggshell protein skeleton, our amino acid analysis did not reveal its presence. Lysine, an abundant amino acid of p48, was average in eggshell (6.8%) and tyrosine, another important amino acid of

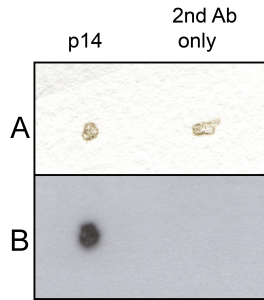


Figure 2.7: Immunoblot of eggshell of *Schistosoma mansoni* with anti-p14. Dot blot of eggshell protein skeleton. The dotted eggshell fragments are shown in A, the chemiluminescent signal for p14 or from Horseradish peroxidase (HRP) conjugated anti-hamster IgG (Abcam, Cambridge, UK) as secondary antibody (Ab) only was captured on hyperfilm (B).

p48 could hardly be detected. Both of these amino acids are involved in quinone tanning and this process renders them unrecognizable as lysine and tyrosine.

Immune reactivity of the eggshell proteins

A number of the identified eggshell proteins such as GST, enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are known to elicit an antibody response upon infection [122]. Fig. 2.8 shows how GST and enolase, two of the identified eggshell proteins, stained positive on Western blots when incubated with sera from infected hamsters. Sera were taken from infected hamsters at 2, 4 and 6 weeks of infection. A positive stain for enolase was detected at 4 weeks of infection, which increased at 6 weeks of infection, when GST was also detected. In addition, antibody reactivity against SEA and AWA was tested. Like enolase, SEA started to stain positive at 4 weeks of infection and the signal increased at 6 weeks of infection. Antibody reactivity against AWA started earlier, at 2 weeks of infection and also increased during the course of the infection. Note that at 4 weeks of infection, worms do not produce eggs yet but enolase, a protein we demonstrated to also be present in eggshells, is already recognised by the serum. This demonstrates that enolase is exposed or secreted by developing worms before this protein is presented to the host via incorporation in the *Schistosoma* eggshell.

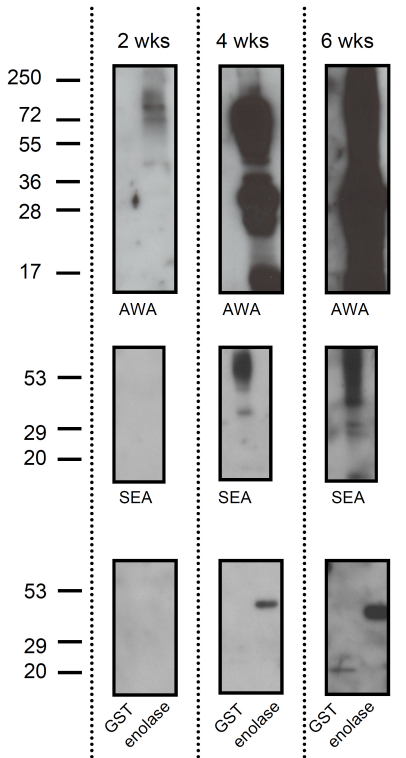


Figure 2.8: Antibody reactivity in hamsters during infection with *Schistosoma mansoni*. Adult worm antigens (AWA), soluble egg antigens (SEA) or protein solutions of *Schistosoma* GST (28 kDa) or rabbit enolase (47 kDa) were run through a SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with sera from infected hamsters at different time points (2, 4 and 6 weeks) during infection as indicated above the blots.

2.4 Discussion

The eggshell is the site of direct interaction between the highly immunogenic eggs of *S. mansoni* and the immune system of its host. Eggs are known to excrete immunogenic proteins [30][110], but it is likely that the proteins that form the eggshell also cause an immunological reaction. The exact protein composition of this rigid cross-linked structure was unknown. The aim of the current study was to identify the proteins that make up the cross-linked eggshell, the outer surface of the egg that interacts with the immune system.

In samples of the purified protein skeleton of eggshells we identified a collection of proteins, similar to those found in other proteome studies of *S. mansoni* [16, 30, 40, 110, 184]. The variety of proteins we detected seems rather random (Table 2.1). Included are many enzymes involved in various metabolic processes, but for instance enzymes involved in crosslinking of the eggshell were not detected.

Surprisingly, we also could not identify by MS either one of the putative eggshell proteins p14 and p48. We have two explanations for this. First, p14 and p48 are tyrosine-rich proteins, which means that it is likely that most of the peptides that are obtained after trypsin digestion will contain one or more tyrosines that have been modified to form cross-links in the quinone tanning process. Thus, tyrosines will not be detected and different side chains will add unknown and variable masses to the peptides which then cannot be identified by peptide mass fingerprinting. Second, *in silico* trypsin digestion of these putative eggshell proteins reveals that trypsin digestion leads to many very short peptides, which are very similar to each other (not shown). This is especially the case for p48, where trypsin digestion results in many pentapeptides of which peptide masses are under the detection limit of the mass spectrometer. Even when detected, such small peptides will not reach a 99.0% probability score for protein identification as they are too short to be specific and thus these peptides will not identify a protein using our criteria for protein identification. It will therefore be impossible to identify these putative eggshell proteins by LC-MS/MS, even if they are abundantly present in the eggshell.

Although p14 could not be identified in our MS analysis, using immunostaining we demonstrated the presence of this bona fide eggshell protein in purified eggshell fragments (Fig. 2.7). It is not just only present, but the extremely high glycine content (36%) of purified eggshell fragments detected by amino acid analysis indicates that p14 contributes largely to total eggshell. The glycine content of eggshell, of the identified proteins and of p14 enabled us to make a general estimation of this contribution of p14 to total eggshell. In the proteins that we identified by MS, average glycine levels were 8.2% of the 17 analysed amino acids, ranging from 4 to 25%. In p14, 47% of the amino acids were glycine. This leads to the following equation: $[p14] \times 47 + [\text{rest}] \times 8.2 = 36$, where $[\text{rest}] = 1 - [p14]$, which demonstrates that p14 contributes to total eggshell for approximately 70%.

The set of proteins that contribute to the eggshell for the other 30% seems to be rather random. However, its average glycine level is significantly higher (7.7%) than the average of all 13,529 *S. mansoni* proteins available on Uniprot (4.8%) ($P < 0.001$), while tyrosine (3.1% versus 3.4%) and lysine levels (6.8% versus 6.0%) are similar. Although glycine levels in identified eggshell proteins do by no means approach that of total eggshell or p14, its elevated levels in the identified eggshell proteins are remarkable. Apparently, there is a preference for proteins with higher glycine levels during cross-linking, although glycine does

not contribute to quinone tanning.

While our analysis of the amino acid composition of eggshell indicated that p14 is present in the eggshell, such an analysis could not be used to estimate the presence of p48. Although p48 is rich in tyrosine, lysine and aspartic acid, none of these amino acids was abundant in our samples. As the amount of p48 in eggshell is known to be much less than p14 [33, 93, 102], tyrosine, lysine and aspartic acid levels of p48 are not abundant enough to feature in a mixed sample of total eggshell proteins. Furthermore, lysine and tyrosine that have been modulated to form cross-links by quinone tanning could not be traced back in the amino acid analysis after hydrolysis, resulting in very low detection of these amino acids.

The majority of the eggshell proteins identified by MS were non-structural proteins. Although these proteins were unexpected, immunoscanning microscopy of HSP70 validated our results. The identified proteins are not specific for the eggshells. Many identified proteins such as actin, tubulin, p40, HSP70 and glycolytic enzymes have also been identified by Cass *et al.* (2007)[30] as proteins secreted by *S. mansoni* eggs. In contrast, Mathieson and Wilson (2010)[110] found that these proteins are not present in secretions of eggs. Secreted or not, it is not likely that our samples were contaminated with proteins secreted by eggs because our eggshell fragments were cleaned extensively before proteomic analysis. The absence in our samples of proteins secreted by eggs is confirmed by the absence of IPSE/alpha-1 and omega-1, the two major proteins secreted by eggs. Similarly, contamination of our purified samples with other proteins originating from Von Lichtenbergs envelope, Lehman's lacuna or Reynolds' layer is just as unlikely due to the extensive pre-treatment.

The proteins identified in our purified fraction (Table 2.1; Fig. 2.7) are largely comparable with those of other egg fractions and even with those of other developmental stages of the parasite [16, 40]. The best explanation for the presence of these abundant cellular proteins, originating from surrounding vitelline cells, is that they happened to be around at the site and time of eggshell synthesis and were cross-linked to the major eggshell proteins. The absence of the two most abundant proteins secreted by eggs, IPSE/alpha-1 and omega-1, gives an indication of the timing of the cross-linking process. After production of the egg further maturation occurs, as the egg excreted by the female worm is still undeveloped and consists of an ovum and vitelline cells surrounded by the eggshell. Our results thus indicate that the actual formation of the eggshell including cross-linking of the proteins is most likely finished before the miracidium starts secreting proteins.

Although the observed incorporation of proteins originating from neighbouring vitelline cells may seem to be an unintended feature of eggshell production, the presence of these proteins may have immunological consequences. Many of the proteins we identified as part of the eggshell protein skeleton are known schistosome antigens such as p40, phosphoenolpyruvate carboxykinase (PEPCK) and thioredoxin peroxidase. These proteins induce cellular responses [3, 200] or antibody responses [122]. The immunogenicity of these usually intracellular proteins can now be explained as these proteins are part of the eggshell. As a consequence of these immunogenic properties, some of the eggshell proteins identified, such as GST and GAPDH, were proposed as vaccine candidates. However, the success of these vaccines in preventing infection was limited [133]. This might now be explained by the fact that the vaccines target the eggs where the proteins are exposed and not the actual infection by the adult worms. In fact, vaccination with such antigens might even induce pathology as it enhances the immune response against the eggs, thereby enhancing granuloma formation and disease symptoms.

Altogether, our results demonstrate that the eggshell is not only composed of the specific eggshell proteins p14 and p48, but also includes proteins available at the site of eggshell production. Therefore we propose a new model of the *Schistosoma* eggshell. The main component of the eggshell is the putative eggshell protein p14. It makes up the majority of the eggshell. However, at the same time other proteins, whichever are available at the place and time of eggshell production, are also incorporated, albeit to a much lesser extent. Cross-linked together, all of these proteins make up a very rigid structure to protect the developing miracidium.

Acknowledgements

We thank Professor Phil LoVerde (University of Texas Health Science Center, San Antonio, Texas, USA) for providing us with the anti-p14 antibodies and for valuable comments on the manuscript. Niels Bohnen and Marion Schmitz are thanked for their contributions to experiments on the purification of eggshell.

Chapter 3

Schistosoma mansoni: The egg, biosynthesis of the shell and interaction with the host

Saskia deWalick¹
Aloysius G.M. Tielens¹
Jaap J. van Hellemond¹

Experimental Parasitology 2012, 132:7–13.

¹ Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands

Abstract

The schistosome eggshell is a hardened and tanned structure made from cross-linked proteins. It is synthesized within the female worm from many different kinds of proteins and glycoproteins. Once the egg is released in the circulation, the outer surface of the eggshell is exposed and hence a direct site of interaction between the parasite and the host. The major eggshell protein is p14, but about one third of the eggshell is made from common cellular proteins, some of which are known to be immunogenic. This has many consequences for parasite-host interactions. However, so far, the eggshell has gained little attention from researchers. We will discuss the structure of the eggshell and its role in granuloma formation, host factor binding and egg excretion.

3.1 Introduction

Production of the *Schistosoma* egg

Schistosomes are digenetic parasitic flatworms. The female *Schistosoma mansoni* excretes around 350 eggs daily which equals to approximately one egg every 5 min [32]. The oocyte is produced in the ovary and released in the oviduct (Fig. 3.1). Here it is fertilized by sperm that comes from the sperm reservoir, a dilated region in the oviduct. The fertilized oocyte moves further along the oviduct, which joins the vitelline duct. Here, 30–40 vitelline cells originating from the vitelline gland surround the fertilized oocyte [175]. Together, the fertilized oocyte and its surrounding vitelline cells move to the ootype (Fig. 3.2a), thereby passing Mehlis' gland. The actual function of this structure remains elusive. Several functions of the Mehlis' gland have been suggested. It is proposed to lubricate the uterus for the passage of the egg or to activate spermatozoa. Other suggested functions of Mehlis' gland are involved in eggshell formation: release of eggshell granules, control or initiate cross-linking of the eggshell or provide a membrane which serves as a template on which the proteins accumulate to form the eggshell [174]. Once in the ootype, contractions of the ootype make the vitelline cells release their granules which contain eggshell precursor proteins (Fig. 3.2b). The eggshell formation starts here. The eggshell is shaped by the ootype and strengthened through tyrosinase activity that causes cross-linking of the released eggshell precursor proteins (Fig. 3.2c and d). The egg is now ready to pass the uterus and to be released in the circulation, where the miracidium further matures within the eggshell (Fig. 3.2e).

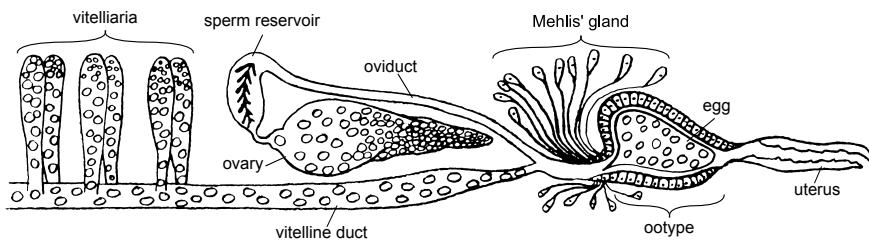


Figure 3.1: Schematic drawing of the female reproductive tract of *Schistosoma mansoni*. After Gönert(1955) [72] and Smyth(1966) [173].

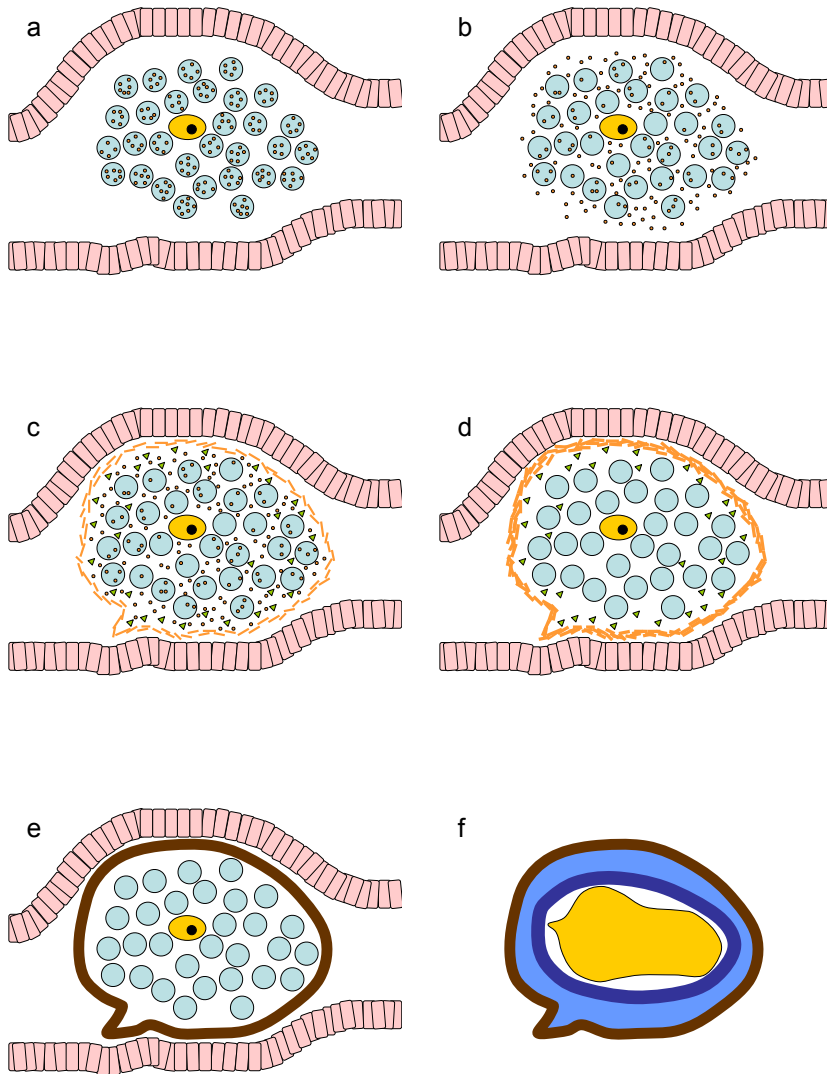


Figure 3.2: Formation of the eggshell in the ootype. Scheme of eggshell formation in *Schistosoma mansoni*. In pink the ootype, yellow the fertilized oocyte, light blue circles are vitelline cells with vitelline droplets (orange) containing eggshell precursor proteins, green triangles are tyrosinases. In the mature egg (f) the miracidium (yellow) is surrounded by Von Lichtenberg's envelope and Reynolds' layer (blue layers) and the eggshell (brown).

Maturation of this newly produced egg of *S. mansoni*, that simply consists of the cross-linked eggshell surrounding the ovum and vitelline cells, takes about a week. In this period the vitelline cells provide nutrients for the developing miracidium, which also obtains nutrients from the host [6]. Newly deposited eggs lack complex subshell structures between the eggshell and the embryo, but these appear during the development of the egg [6, 123]. First, in an early stage, a few cells detach from the embryo and form a thin syncytial layer, known as “von Lichtenberg’s envelope”, between the eggshell and the developing miracidium [6, 123]. As development proceeds, this envelope becomes thicker, completely encloses the egg contents, the embryo and vitelline cells and separating them from the shell of the egg. This nucleated envelope contains extensive rough endoplasmic reticulum indicating that this is a place of active protein synthesis [6]. During the further development a new layer of extra cellular material is formed between von Lichtenberg’s envelope and the eggshell, called Reynolds’ layer, which mainly consists of granulated material most likely originating from the envelope [6, 123]. The envelope and not the miracidium produces the proteins secreted by the egg, including IPSE/alpha-1 [109, 162].

Eggs are produced one by one by female *S. mansoni* and the next egg will be produced once the previous one has been released. With an egg production of 350 per day, female schistosomes can be considered as true egg factories. This is reflected in the fact that about 10% of total worm mRNA encodes for one eggshell protein, p14 [128, 170].

3.2 Structure of the eggshell

Cross-linking and tyrosinase activity

The eggshell is a hardened and tanned structure made from cross-linked proteins. The cross-linking process is known as quinone tanning and occurs by formation of quinone bonds. It is dependent on tyrosinase activity. Tyrosinases are copper-containing glycoenzymes that can catalyze both the hydroxylation of mono-phenols (tyrosine residues) to *ortho*-diphenols (L-dihydroxyphenylalanine, L-DOPA) and the subsequent oxidation of this L-DOPA to *ortho*-quinone (Fig. 3.3). In this way, accessible tyrosine residues of proteins are converted to *o*-quinones. These *o*-quinones are very reactive compounds and can form adducts by reaction with nucleophilic compounds, such as free amino or sulfhydryl groups on adja-

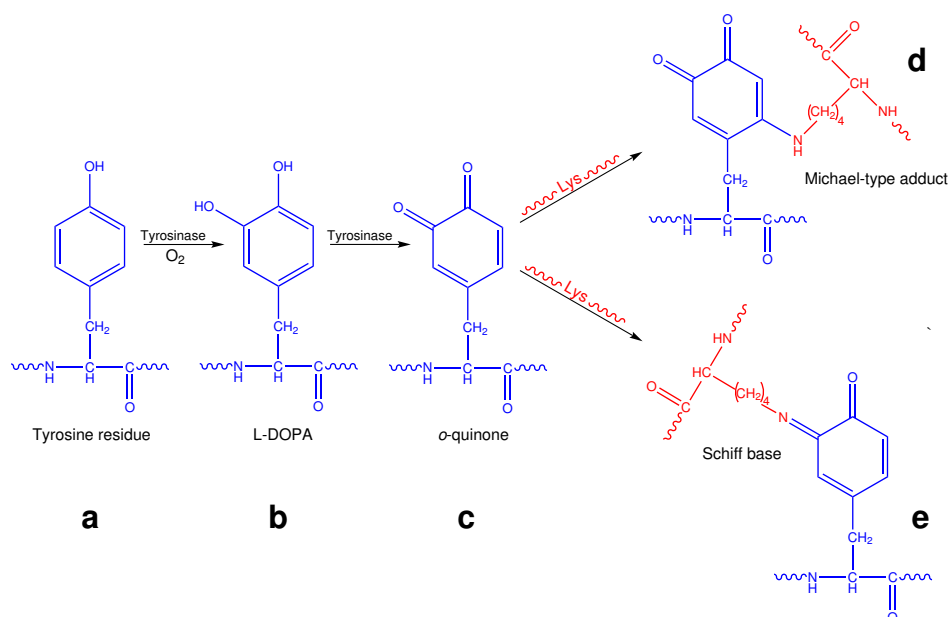


Figure 3.3: Quinone tanning. Tyrosinase converts tyrosine residues (a) into L-DOPA (b) and subsequently L-DOPA residues into *o*-quinones (c). These very reactive *o*-quinones can form adducts by reaction with nucleophilic compounds, such as free amino groups of lysine residues on adjacent proteins by the formation of either N-quinonyl derivatives (Michael-type adduct) (d) or Schiff bases (e).

cent proteins. These reactions are known to occur in processes as diverse as the browning of foods and plant leaves, and the sclerotization and tanning of insect cuticles [11]. Nowadays it is known that in plants and insects the formation of these quinone adducts occurs via two different reactions of free amino groups with the *o*-quinones: the formation of N-quinonyl derivatives (Michael-type adducts) and of Schiff bases (Fig. 3.3). Although it was originally suggested that in *S. mansoni* the cross-linking of the eggshell occurs via formation of the N-quinonyl adducts only [174], it is in our opinion more likely that also in helminths, the eggshell tanning occurs via both processes (Fig. 3.3).

This cross-linking within a single protein and between neighboring proteins, results in an extensively cross-linked protein matrix. The appearance of the *o*-quinones leads to a rapid change in color and hardening of the proteins, rendering them intractable to all protease activity and only hydrolytic treatments can dissolve the cross-linked eggshell [194]. The cross-linked eggshells are highly flu-

orescent. Their excitation/emission spectra are similar to the spectra of products formed by mushroom tyrosinase in a mixture of lysine with L-DOPA, indicating that in the eggshell lysine is the most probable amino acid that forms quinone bonds with tyrosine (see below) [165].

Tyrosinase activity appears to be developmentally regulated with peak transcription in mature female worms. Enzymatic activity is specifically located in the vitelline cells [56, 65]. Eggshell formation by tyrosinase activity is pH dependent and triggered by alkaline environment [195].

Specific eggshell proteins

Putative eggshell proteins have been identified in the late 80s by searching for specific mRNAs that are only expressed in mature females and not in males, nor in immature female worms, miracidia or cercariae. The corresponding genes could either encode eggshell precursors or proteins involved in the synthesis of the eggs and eggshell. Interestingly, tyrosinases, which are known to be developmentally regulated and involved in eggshell synthesis, have not been identified this way. This suggests that the catalytic enzymes required for eggshell formation are not abundantly expressed when compared to structural proteins. In addition, eggshell proteins that are not exclusively produced in mature females can not be detected this way.

The first putative eggshell protein to be described was p14 [13, 14, 93, 94, 150, 169]. There are several copies of the p14 gene in the genome of *S. mansoni*. They do not rearrange, and they are not sex linked [14, 169]. The p14 gene is developmentally regulated and only expressed in mature female worms. The p14 gene is only expressed in mature vitelline cells in the vitelline duct and within the egg enclosed by the eggshell in the ootype. Transcripts of p14 are undetectable in RNA obtained from eggs from the liver [33]. The p14 gene is the most abundant mRNA transcript in female worms and accounts for 10% of the mRNA of the entire organism. Even in samples of mixed sex, this mRNA is by far the most abundant transcript [128]. The p14 protein is present in all the vitelline droplets of vitelline cells in mature female worms [93]. Immunoblots confirmed the presence of p14 in eggshell [47].

Although less abundant, p48, another putative eggshell protein, shows a similar expression pattern when compared to p14. However, the amount of p48 mRNA in worm is 10–30-fold less abundant than that of p14 [33]. *In vitro* translation of the

cDNA with [³H]tyrosine produces a 48 kDa protein. A protein of the same size is also present in translation products of mRNA from mature female worms, but not in that of male or immature female worm [88]. Antibodies raised against recombinant p48 proteins recognize a 48 kDa protein on Western blot in homogenates of mature female worms, whereas no signal can be detected in homogenates of male worms or immature female worms. In addition, antisera raised against mature female worm extract recognize recombinant p48 proteins, while antisera raised against male or immature female worm extracts do not [33].

Reis *et al.* (1989)[144] described another female specific mRNA (FS800), that is only expressed in vitelline cells of mature female worms. The mRNA sequence was described to comprise two Open Reading Frames (ORFs) in two distinct reading frames. The longest ORF with the starting codon upstream of the alternative ORF is currently annotated as the translated protein sequence in the *S. mansoni* genome [9]. However, to our knowledge, there are no data available yet on the translation of this female-specific mRNA into protein(s).

Finally, one other female specific protein has been described by Menrath *et al.* (1995)[117] and was described as mucin-like. Later, it was renamed p19 [119] but it has now been annotated as eggshell protein (Q26570_SCHMA) in the UniProt database. However, expression of the gene can be found in the epithelium surrounding the female oviduct close to its entrance into the ootype, but not along the vitelline duct and neither in ovary, vitellarium or ootype [117]. Although this protein is female specific, this is apparently not an eggshell protein.

Non-specific eggshell proteins in the eggshell

Recently, the protein composition of purified eggshell fragments was investigated by a proteomic approach [47]. This study showed that the eggshell is not only composed of the above mentioned specific eggshell proteins. About one third of the proteins in the eggshell are common cellular proteins. Among them are known schistosome antigens such as major egg antigen p40 and HSP 70, but also structural cellular proteins (actin and tubulin) and non-structural proteins of which many are glycolytic enzymes [47]. Many of these non-specific proteins in the eggshell have also been identified inside the egg [110], in adult worm tegument [17, 184] and in schistosomula and cercariae [40].

The list of non-specific eggshell proteins may not be complete, as proteins that are cross-linked are not always detectable by mass spectrometry [47]. Al-

though the incorporation of these non-specific eggshell proteins may be an unintended feature of eggshell production, the presence of these proteins, even in small amounts, may have immunological consequences. Many of them elicit a cellular or humoral immune response in the host upon a schistosome infection. Some of the incorporated proteins, such as glutathione-S-transferase (GST) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were proposed and tested as vaccine candidates, albeit with little success in preventing infection [133]. Vaccination with such antigens might actually induce pathology as it enhances the immune response against antigens in the eggshell, thereby enhancing granuloma formation and disease symptoms.

It should be noted that IPSE/alpha-1 and Omega-1, the two major egg secretion proteins (ESP), have not been found among the proteins of the eggshell [47]. This indicates that the proteins excreted by eggs are not incorporated into the shell of the egg, which suggests that cross-linking of the eggshell proteins is finished before excretion of proteins by the egg begins.

Amino acid composition of the eggshell and eggshell proteins

In p14, 44% of the amino acids are glycine. This protein has some homology with the carboxyl terminal of mouse cytoskeleton keratin and with silk moth chorion proteins [13]. The other major eggshell protein, p48, has only slightly elevated glycine levels, but contains high levels of lysine and aspartate, and its most abundant amino acid is tyrosine. The other proteins we identified as part of the eggshell do not have extreme levels of glycine, although their average glycine content is significant higher than the average glycine content of *S. mansoni* proteins [47].

The amino acid composition of fluorescent granules in the vitelline cells is highly similar to that of hydrolyzed eggshells, suggesting that all the reagents for eggshell formation are present in the vitelline cells [195]. Hydrolyzed total eggshells revealed that 36% of the amino acids are glycine, which hereby largely attributes to the eggshell [24, 47, 150]. The high glycine levels in the analysed eggshell hydrolysates suggest that p14 widely contributes to the eggshell. This contribution was estimated to be 70%. Immunoblots with anti-p14 antibodies showed that p14 is indeed present in eggshell [47]. The role of the high glycine content of p14 and total eggshell remains elusive, but a high glycine content is associated with coiled coils. Rodrigues *et al.* (1989)[150] proposed a structure

model of p14 based on its amino acid sequence and the high glycine content. In this structure, the protein is composed of short anti-parallel beta strands in which glycines and other residues with small side chains lie within the strands and tyrosines are arranged at the bends. This way, tyrosine residues are still available for cross-linking.

As tyrosine is essential in quinone tanning, eggshells are expected to contain high levels of this amino acid too. This is indeed the case for the two main eggshell proteins p14 (11.3%) and p48 (26.1%). However, little tyrosine can be measured in hydrolyzed eggshells or granules. This is probably not because of the absence of tyrosine, but due to the changes of the tyrosine molecule upon cross-linkage during eggshell formation, which makes them no longer recognizable as tyrosine (Fig. 3.3). Although little tyrosine could be detected in eggshell hydrolysates or fluorescent granules, radiolabeled tyrosine was efficiently incorporated in eggshell when injected in infected mice, indicating that eggshell does contain significant amounts of tyrosine [24].

A third amino acid that is expected to be abundant in eggshell is lysine, as this amino acid is likely to be the residue that forms the cross-link with the *o*-quinones [165]. Indeed, the eggshell proteins p14 and p48 contains high levels of lysine (5.6% and 17.1%). However, no abundant levels of lysine could be detected in total eggshell. As with tyrosine, this may be due to cross-linking of the lysine residues to the *o*-quinones.

Glycans

The eggshell does not only consist of cross-linked proteins, but on these proteins, schistosome specific glycans are present. Composition analysis of glycans was suggestive for mannose and fucose residues and the presence of N-linked glycans, but additional O-linked glycans and other glycan classes may also be present. The glycan epitopes F-LDN(-F) and Lewis X are present on eggshell, as demonstrated by immunoblots [47]. These glycans are known to be immunogenic, but little is known about the molecular basis of glycan-mediated host-parasite interactions [186].

3.3 Interaction with the host

Egg secretion proteins (ESP)

Although the eggshell is a direct site of interaction between the parasite and the host, little research has focused on this tightly cross-linked and hard to analyze structure. Rather, much research has focused on ESP. Secretion of ESP by the miracidia starts a few days after the eggs have left the female worm [6]. There have been some contradictory papers reporting that ESP contains either a wide range of proteins [30] or only a few [6, 110]. There is, however, consensus that the major ESP are IPSE/alpha-1 and Omega-1. The total ESP and isolated or recombinant IPSE/alpha-1 or Omega-1 have widely been investigated for their capacity to drive a strong Th2 response. This immune response protects the host from fatal disease. However, this Th2 response is a strong inducer of granuloma formation [134]. Furthermore, T-cell responses are essential for excretion of eggs. Experiments in mice have shown that in the absence of T-cells, egg excretion from the body is greatly hampered [49]. In humans, HIV patients with low CD4+ T-cell count have reduced numbers of egg excretion [90].

Granuloma formation and the eggshell

While many investigators have focused on ESP, much less research has been done regarding the eggshell. It has recently been shown that the eggshell contains numerous other proteins and glycoproteins besides the major eggshell proteins. Many of these other proteins and glycoproteins are known schistosome antigens, such as p40, phosphoenolpyruvate carboxykinase (PEPCK) and thioredoxin peroxidase. These antigenic proteins induce cellular responses [4, 200] or antibody responses [122]. Serum of patients infected with schistosomes contains antibodies that recognize purified eggshells, from which all not covalently bound compounds were removed [47] (Fig. 3.4a), whereas these were not present in control serum (Fig. 3.4b). It is thus evident that not only ESP interact with the immune system, but also the eggshell itself is recognized by antibodies. Furthermore, eggs isolated from livers of infected hamsters are covered by host antibodies (Fig. 3.4c).

It is known that Th2-cells play a role in granuloma formation. Likewise, antibodies bound to the eggshell may enhance pathology. Since the eggshell is resistant to protease activity, and because the incorporated antigenic proteins in the eggshell bind host antibodies, the eggshell surface will form an indigestible

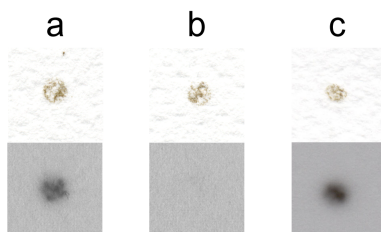


Figure 3.4: Antigenicity of eggshells. Purified eggshell skeletons [47] were incubated with serum from a human schistosomiasis patient (a) or control serum (b) and subsequently with an HRP conjugated anti-human IgG secondary antibody. Eggs freshly isolated from livers of infected hamsters were incubated with an HRP conjugated anti-hamster IgG antibody (c). Eggs were subsequently dotted on a Whatman paper (top panel) and the chemiluminescent signal of these dots was captured on light sensitive film (bottom panel).

antigen-antibody complex. These structures are known to induce chronic inflammatory responses that can lead to granuloma formation by a complex interaction between CD4⁺ T-cells, macrophages and cytokines [201]. Also ESP are involved in granuloma formation around the egg [110, 134].

Activation of T-cells and binding to antibodies may also play a role in the process of extravasation through the endothelium and the gut wall. *Ex vivo*, antibodies bind to ESP that exit the egg through pores in the eggshell. This is known as the circumoval precipitin (COP) reaction. At the point of secretion, a precipitate will form. Globular blebs or septate precipitates appear on the wall of the schistosome eggs after incubation with the serum of infected individuals [66].

How to get out of the host?

The egg is not capable of moving by itself. Thus, all movement has to be induced by external forces. First it is released into the circulation by the female worm; next the circulating blood will take hold of the egg. However, the intended pathway for the *S. mansoni* egg is not to follow the blood stream, but to extravasate the vessel wall and penetrate the gut wall in order to get out with the feces. This process takes at least six days, but may also take weeks [89]. It is unlikely that the female worm can push such an enormous egg through the host structures. Therefore, the egg probably attracts host proteins and host cells in order to hold onto the vessel wall and help passage through the endothelium and the gut wall, allowing escaping the host and continuing the life cycle.

In addition to the widely studied schistosome-specific glycoproteins IPSE/alpha-1 and Omega-1, some house-keeping enzymes have been described to be virulence factors. Enolase and GAPDH, proteins that have also been identified as part of the eggshell, are such house-keeping enzymes which are also virulence factors in *Schistosoma* and other organisms. They both act as surface associated receptors that bind plasminogen, thereby capturing fibrinolytic activity of the host on the eggshell surface [206]. This activity represents a mechanism to enhance virulence possibly required for tissue invasion and may thereby facilitate the escape of the eggs [132]. Enolase in other pathogens has also been shown to bind actin and fibronectin. Fibronectin is a main component of extracellular matrix surrounding epithelial and endothelial cells. Blocking enolase decreased adhesion of other pathogens to endothelial cells [10, 57]. By binding to and activation of host factors, the eggshell may thus play an important role in getting out of the host. Thrombocytopenia significantly reduces egg excretion [125]. Platelets massively adhere to eggshells of *S. mansoni*, resulting in activation of platelets [203]. Activated platelets generally activate endothelial cells and may thereby facilitate passage through the endothelium. The eggshell may thus play an important role in getting out of the host by binding to and activation of host factors like plasminogen and platelets. Additionally, escape from the host may be facilitated by proteolytic activity of eggs and miracidia or from other (host) proteins and cells attracted by the egg [138, 172].

Acknowledgments

Production of Fig. 3.3 by Michiel Bexkens is gratefully acknowledged. We sincerely thank the two anonymous reviewers for their helpful comments.

Chapter 4

Binding of von Willebrand factor and plasma proteins to the eggshell of *Schistosoma mansoni*

Saskia deWalick¹
Paul J. Hensbergen²
Michiel L. Bexkens¹
Christina Grosserichter-Wagener¹
Cornelis H. Hokke³
André M. Deelder²
Philip G. de Groot⁴
Aloysius G.M. Tielens¹
Jaap J. van Hellemond¹

International Journal for Parasitology 2014, 44:263–268.

¹ Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands

² Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands

³ Department of Parasitology, Center for Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

⁴ Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, The Netherlands

Abstract

Schistosoma mansoni eggs have to cross the endothelium and intestinal wall to leave the host and continue the life cycle. Mechanisms involved in this essential step are largely unknown. Here we describe direct binding to the *S. mansoni* eggshell of von Willebrand factor and other plasma proteins involved in haemostasis. Using deletion-mutants, we demonstrated that it is the A1 domain of von Willebrand factor that binds to the eggshell. Our results suggest that binding of plasma proteins to the eggshell promotes binding to the endothelium, initiating the passage of the egg through the blood-vessel wall to be excreted in the end.

Schistosoma spp. are helminths causing the tropical disease schistosomiasis. An estimated 200 million people are affected, most of them living in sub-Saharan Africa [74]. These parasites penetrate their human host through the skin after which the cercariae transform into schistosomula that subsequently enter the circulation. In the case of *Schistosoma mansoni*, male and female worms meet in the portal vein, where they pair and mature before moving to the mesenteric veins, their final habitat [74]. Mature female worms excrete approximately 300 eggs per day, which is about one every 5 minutes [32]. In order to complete the schistosome life-cycle, the eggs have to be excreted with the faeces. Therefore, eggs have to cross the blood-vessel wall as well as the intestinal wall. Many eggs fail to extravasate and are transported by the blood flow to the liver where they get stuck and induce granuloma formation, fibrosis of the liver and portal hypertension. These non-excreted eggs are the major cause of morbidity in schistosomiasis.

Eggs have a rigid shell structure of cross-linked proteins and thus cannot propel themselves [48]. Therefore, eggs depend on external forces in order to cross the host tissues. Mechanisms involved in excretion of eggs are largely unknown, even though this is an essential step in the life-cycle of schistosomes. Eggs of several schistosome species have a sharp spine and it could be imagined that the presence of this spine aids in passing through the obstructing structures of the host [89]. However, eggs of *Schistosoma japonicum* and *Schistosoma mekongi* lack a sharp spine, which makes this theory unrealistic. It is known that the process of excretion is partly dependent on the host immune reaction directed towards eggs [49], but additional mechanisms are likely to be involved.

It has been demonstrated that eggs attach to endothelium and that endothelial cells actively migrate over the eggs [64]. The endothelial surface-adhesion molecules, ICAM-1, E-selectin and VCAM-1, and surface exposed Lewis X glycans on *S. mansoni* eggs play an important role in egg adhesion to the vascular endothelium [98]. In addition, plasma factors enhance egg attachment to endothelial cells *in vitro* and platelets have a role in extravasation and excretion *in vivo* [64, 125, 126]. Furthermore, schistosome eggs were also shown to be a potent and direct activator of platelets and platelets were shown to adhere en masse to eggs *in vitro* [125, 203]. Activated platelets aggregate and stimulate secondary coagulation (the plasma clotting factor cascade) which results in fibrin fibre formation to stabilise the platelet plug. Apparently, there is an interaction of the schistosome eggshell

with the host haemostatic system, but the molecular structures involved in the activation of the haemostatic system are unknown.

We hypothesised that the egg probably attracts host proteins and host cells in order to hold onto the vessel wall and help passage through the endothelium and the gut wall, allowing excretion from the host and continuation of the life cycle. Therefore, we analysed the interaction of *S. mansoni* eggshells with von Willebrand factor (VWF), as this clotting factor is crucial for anchoring clotting material to damaged or activated surfaces of the blood vessel, and VWF could thus play an essential role in egg extravasation. VWF is an adhesive glycoprotein of 250 kD which forms multimers that can be extremely large and consist of over 80 subunits, resulting in a multimer of over 20 mega-Dalton. VWF contains many binding domains that can connect platelets to clotting factors and injured surfaces of the endothelium [105, 159]. VWF binds platelet glycoprotein Ib (GPIb) on platelets which are thereby activated and initiate clot formation.

The presence of VWF on *S. mansoni* eggs isolated from collagenase digested livers of infected hamsters was demonstrated by immunofluorescence (Fig. 4.1A). As these eggs were directly obtained from perfused livers, this demonstrates that VWF binding occurred *in vivo* in the blood vessel.

Schistosome eggs were also incubated *in vitro* with purified human VWF. We used both untreated eggs and stripped eggs. From stripped eggs, all non-covalently bound proteins were removed by a procedure which includes multiple washes with high salt conditions, detergents, urea and even boiling in SDS, in order to be entirely sure that no host factors remained on the surface of the schistosome egg [47]. Both untreated (Fig. 4.1C) and stripped eggs (Fig. 4.1D) bound purified human VWF *in vitro*, demonstrating the direct interaction between VWF and the eggshell. The binding of VWF to eggs could also be demonstrated at a more quantitative level using stripped eggshell fragments and enhanced chemiluminescence (ECL) measurements (Fig. 4.1H).

We also incubated stripped schistosome eggs for 1 min with citrate plasma from healthy adult human volunteers, obtained after informed consent. Binding of VWF to eggs was then demonstrated by immune fluorescence (Fig. 4.1F and G). This result demonstrated that VWF in the presence of all other plasma proteins still binds to the schistosome eggs, although the punctuated fluorescence pattern suggests that other plasma proteins compete for egg binding, or that other plasma proteins shield bound VWF prohibiting its detection by immune fluorescence. Altogether these results demonstrate that there is a direct interaction between

schistosome eggshells and VWF. To determine which VWF domain is involved in eggshell binding, we incubated eggs with recombinant human VWF of which either the A1 [97], A2 [95] or A3 domain [96] was deleted. Reduced VWF binding was only observed with the A1 domain-deleted VWF, but not with VWF lacking the A2 or A3 domain (Fig. 4.1I). This indicates that VWF binds eggshells with its A1 domain. Domain A1 of VWF is a relatively small part of VWF as it comprises circa 250 amino acids, which is approximately 15% of the entire VWF protein [97]. In addition, deletion of the A1 domain does not affect the structure and function of the other domains in VWF [95–97]. Hence, the dependence on a small domain of VWF for its binding to schistosome eggs suggests a specific interaction.

The A1 domain of VWF also binds GPIb, a glycosylated receptor on platelets. Since the eggshell contains glycan structures as well [47], we hypothesised that eggshell glycans may play a role in the binding of VWF. However, we could not find indications for glycan-mediated VWF binding to eggshells. Treatment of eggshells with glycosidases did not reduce VWF binding (data not shown), but these treatments did not remove all glycan structures either and therefore these experiments were inconclusive. Blocking of glycan access with monoclonal antibodies against schistosome glycans (291-2G3-A, 114-3A5, 128-4F9-A (which bind to Gal β 1-4(Fuc α 1-3)GlcNAc (Lewis X)), 273-3F2 (which binds to GalNAc β 1-4GlcNAc (LDN)) [192] and 114-4D12 (which binds to Fuc α 1-2Fuc α 1-3 (DF) termini) [148]) had no effect on the binding of VWF to eggshells (data not shown). Furthermore, treatment of eggshells with periodate, which disrupts glycan structures, did not reduce VWF binding (data not shown). In conclusion, VWF binds directly to eggshells through its A1 domain, most likely in a non-glycan-mediated manner.

When eggs are laid by the female schistosome in the circulation of the host, they immediately come into contact with circulating plasma proteins. We hypothesised that, next to VWF, other plasma proteins involved in fibrin formation, platelet activation and aggregation, bind to eggshells. To test this, we incubated stripped eggshell fragments with plasma and subsequently washed those six times with PBS. Thereafter, bound plasma proteins were released by boiling the eggshells in Laemmli buffer. Released proteins were analysed by SDS-PAGE together with a lane of diluted plasma (Fig. 4.2). A clear difference in protein band pattern was observed between plasma and plasma proteins bound to schistosome eggshell fragments, especially in the high molecular weight range, demonstrating selective binding of certain plasma proteins to eggshells. Following trypsin digestion and LC-MS/MS analysis, the identified proteins in both samples were compared (Table 4.1

and Supplementary Table S1). The high molecular weight plasma protein which specifically bound to eggshells (gel slices 1 and 2) was identified as fibronectin. Although the pattern of protein bands between approx. 50–75 kDa in plasma was similar to the pattern of proteins bound to eggshells, the major proteins in these gel slices were different. In this respect it is noteworthy that albumin, the major plasma protein, could hardly be detected among the proteins bound to eggshells. This validates the selectivity of the binding. The plasma proteins specifically bound to eggshells in the molecular weight region of albumin consisted mainly of fibrinogen (gel slices 5–7). Fibrinogen is a haemostatic protein and is the precursor of fibrin. When cross-linked, fibrin networks reinforce a platelet plug. Similar to fibrin, fibronectin plays an essential role in wound healing and blood clot formation.

Figure 4.1 (following page): von Willebrand factor binding to *Schistosoma mansoni* eggshells. Eggs of a Puerto Rican strain of *S. mansoni* maintained in Golden hamsters for which animal ethics was approved (licence EUR1860-11709), were isolated 49 days p.i. as described previously [47]. Eggshell fragments were prepared by crushing eggshells in a micro-dismembrator S (Braun Biotech Int., Melsungen, Germany). “Stripped eggs” and “stripped eggshell fragments” were prepared by removal of non-covalently attached host material from isolated eggs or eggshell fragments by consecutive washes with 2 M NaCl in PBS, 1% (v/v) Triton X-100 in PBS, 8 M urea with 0.6% β -mercaptoethanol in PBS, and finally with 1% (w/v) SDS at 95 °C for 30 min [47]. Untreated schistosome eggs isolated from livers were incubated with polyclonal anti-von Willebrand factor rabbit antibody (Dako) (A) or PBS (B) for 1 h. Subsequently, for immune fluorescence microscopy these eggshells were washed with PBS and stained with Alexa 633 conjugated anti-rabbit IgG antibody (Invitrogen, USA). Fluorescence microscope images were taken using Cell[^]F software (Olympus, Germany). Untreated eggs (C) or stripped eggs (D–G) were incubated with 500 μ l of PBS/BSA/Tween (E, G), or PBS/BSA/Tween with purified human von Willebrand factor (0.2 μ g/ml) (C and D), or plasma using citrate as anti-coagulant (F). After incubation, the eggshells were washed three times with PBS and then stained for von Willebrand factor binding as described above using a polyclonal anti-von Willebrand factor rabbit antibody (Abcam, UK). Representative pictures from at least two independent experiments show the immune staining for von Willebrand factor (C–G). To quantify bound von Willebrand factor (H and I), stripped eggshell fragments were incubated with or without purified human von Willebrand factor (H) or with recombinant deletion mutants of von Willebrand factor (I), after which the eggshell fragments were washed with PBS, incubated with anti-von Willebrand factor antibody (Abcam) for 1 h and subsequently incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin antibodies (Dako, Glostrup, Denmark). The eggshell fragments were then incubated in enhanced chemiluminescence detection reagents (Pierce, USA) and dotted on transparent sheets. Chemiluminescence was captured with Alliance 2.7 (Uvitec, UK) and intensity was determined using Image J software. The amount of eggshell material was quantified by measuring scattering at 655 nm (OD 655). von Willebrand factor binding was normalised for the amount of eggshells by expression in arbitrary units ECL/OD655. The fluorescence signal in the presence of added von Willebrand factor was set to 100%; data from 12 experiments (mean + S.D.) (H). Eggshells were also incubated with recombinant deletion mutants of human von Willebrand factor lacking the A1, A2 or the A3 domain, respectively (I). enhanced chemiluminescence signal of full-length von Willebrand factor was set to 100%, data from three independent experiments (mean + S.D.). Magnification in A–E is identical (bar represents 100 μ m) and higher than in F and G (bar represents 50 μ m).

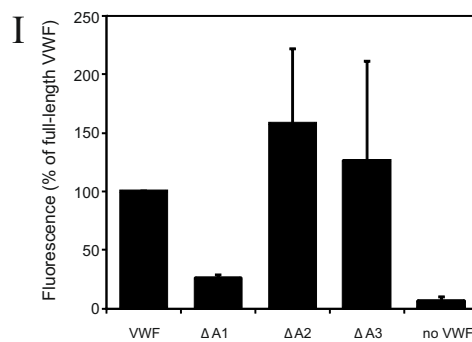
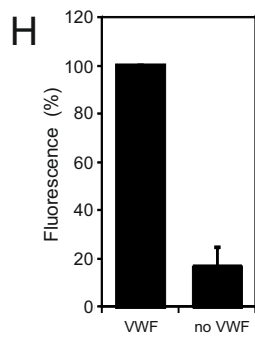
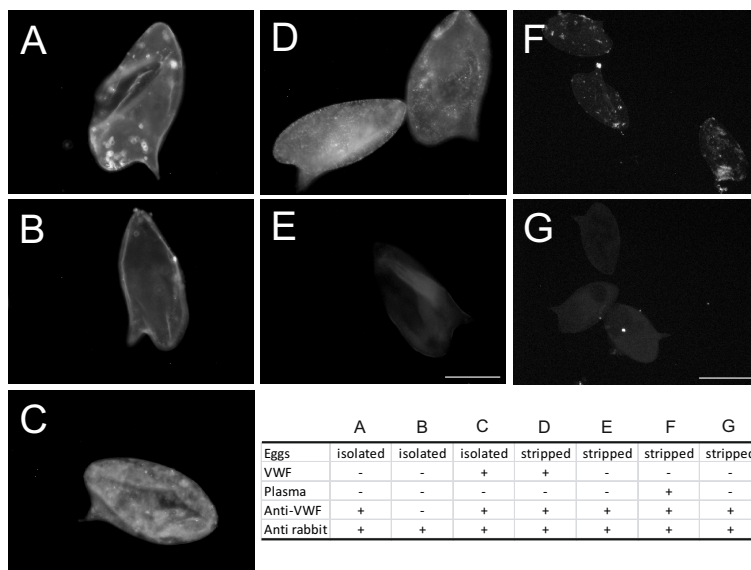


Table 4.1: List of main proteins in human plasma versus proteins of human plasma bound to *Schistosoma mansoni* eggshells. The numbers refer to the gel slices cut from SDS-PAGE as shown in Fig. 4.2. Proteins were in-gel digested with trypsin and subsequently analysed by LC-ion trap MS/MS as described previously [208]. Spectra were searched against the human (UniProt) and schistosome (*Schistosoma mansoni*, release 4.0 h at www.genedb.org) database using the Mascot search algorithm (version 2.2.07). To examine the major differences between protein hits in corresponding bands, only proteins with at least four unique peptides with a peptide score >30 were considered. For each gel slice the most abundant proteins identified are listed, with a maximum of three, ranked by the number of peptides detected for each protein. For a comprehensive overview of the protein identification data, see Supplementary Table S1.

Gel slice number	Total plasma proteins		Eggshell-bound plasma proteins	
	Protein name	Peptides detected	Protein name	Peptides detected ^a
1	Albumin	19	Fibronectin 1	80
2	Albumin	22	Fibronectin 1	139
	Fibronectin 1	13	Talin 1	7
	Alpha-2-macroglobulin	11	-	
3	Albumin	36	Fibronectin 1	34
	Alpha-2-macroglobulin	25	Collagen, type II, alpha 1	5
	Ceruloplasmin (ferroxidase)	11	-	
4	-		Fibronectin 1	9
5	Albumin	64	-	
	Serotransferrin	26	-	
6	Albumin	132	Fibrinogen alpha chain	57
	Fibrinogen alpha chain	15	Vitronectin	14
	Uncharacterised protein	8	Albumin	14
7	Albumin	42	Fibrinogen beta chain	30
	Alpha-1-antitrypsin	28	Fibrinogen alpha chain	23
	Ig gamma-1 chain C region	14	Uncharacterised protein	13
8	-		Fibrinogen gamma chain	32
	-		Fibrinogen alpha chain	18
	-		Actin, beta	7
9	Albumin	19	Actin, beta	27
	-		Apolipoprotein A-IV	12
	-		Fibronectin 1	12
10	Albumin	25	Fibrinogen alpha chain	10
	Haptoglobin	16	-	
	Apolipoprotein A-IV	4	-	
11	Albumin	8	Clusterin	15
	-		Apolipoprotein E	10
	-		Fibrinogen alpha chain	9
12	Albumin	9	Apolipoprotein E	34
	Apolipoprotein E	6	Fibrinogen alpha chain	10
	Fibrinogen alpha chain	6	Fibrinogen gamma chain	10
13	Uncharacterised protein	12	-	
14	Albumin	11	-	
15	Apolipoprotein A-I	11	-	

^a Average of two independent experiments.

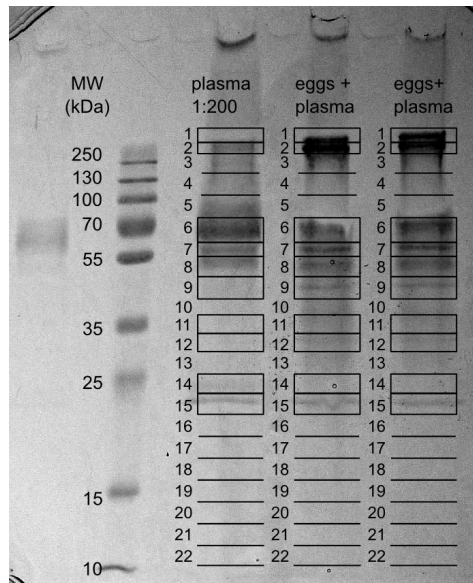


Figure 4.2: Specific human plasma protein binding to *Schistosoma mansoni* eggshells. Blood was freshly drawn from human volunteers in containers containing citrate as anti-coagulant and spun for 10 min at 1200g to obtain platelet-poor plasma. Stripped *S. mansoni* eggshell fragments were incubated for 1 h with 1 ml of this platelet-poor plasma. After incubation, the eggshell fragments were washed six times with PBS, boiled in Laemmli buffer and run through 12% SDS-PAGE (lanes eggs + plasma), with a control lane containing 10 μ l of the used plasma diluted 1:200 in PBS (lane plasma 1:200). Molecular weight marker is shown. Slices were cut from the gel as indicated and numbers correspond to the proteomics data provided in Table 4.1 and Supplementary Table S1.

The set of proteins bound to eggshell was also enriched in actin and talin. These cytoskeleton proteins probably originate from the remaining platelets in the platelet-poor plasma. This is in accordance with previous reports which show that platelets bind very well to eggshells [203]. We also observed an enrichment of apolipoproteins E and A-IV in the set of proteins that bound to eggshells (Table 1). In plasma, Apo-IV is present on chylomicrons, whereas Apo E is present on chylomicrons and on intermediate-density lipoproteins (IDLs). Their presence in the set of proteins bound to eggshells could be a result of binding of lipoproteins to eggshells. It is known that adult worms have a low-density lipoprotein (LDL)-receptor-like protein on their tegument [158] and that the worms bind LDL [34]. The binding of LDL probably helps the adult worms to mask their antigens and hence hide from the immune system. However, on eggshells no LDL-receptor-like protein is known to be present.

In contrast to the immune fluorescence experiments, the proteome analysis of bound plasma proteins did not reveal VWF as a protein bound to schistosome eggs. Fluorescence is a highly sensitive detection method compared with our proteomic workflow. Mass spectrometry detects the most abundant peptides of a trypsin digest of a protein sample, and thus this technique will only show the proteins mostly present in the analysed material. Therefore, absence of detection by this method cannot be interpreted as an indication that the protein in question is not present. Of note, the plasma concentration of VWF (approximately 10 µg/ml) is very low compared with that of fibrinogen and fibronectin (2–4 mg/ml, 0.3–0.4 mg/ml, respectively). These plasma components each bound to the eggshell and were extremely enriched from plasma compared with albumin and other plasma proteins, but the unfavorable concentration ratio of VWF to fibrinogen and fibronectin on the eggshell seems to have left VWF undetectable by proteomics. The near absence of albumin in the proteins bound to eggshells further demonstrates the specificity of the binding of other plasma proteins to *S. mansoni* eggshells. Moreover, the binding efficiencies and kinetics of VWF compared with the other possibly competing eggshell binding proteins identified in our proteomics experiments may also have played a role.

To check whether all eggshell bound proteins were from the host and not from the parasite itself, the mass spectrometric data were also searched against the schistosome database. The only schistosome protein found in this analysis was actin, but all identified peptides showed complete homology to human actin. As no schistosome-specific peptides derived from actin were observed, it is likely that the human plasma and not the schistosome itself is the source of the actin found. Altogether, the set of eggshell-binding plasma proteins is indicative of fibrin formation and platelet binding to eggshell.

Our results demonstrate that the schistosome eggshell binds the plasma proteins, VWF, fibrinogen and fibronectin, which play an essential role in clot formation. The main function of VWF is binding to other proteins by protein-protein interaction, for which VWF comprises many binding domains. Upon injury VWF plays an important role in platelet-vessel wall adhesion by binding both platelets (via GPIb and GPIIb-IIIa) and the extracellular matrix of the damaged endothelium exposing collagen and fibrin. Binding of VWF to the GPIIb-IIIa complex, which is exposed on activated platelets only, also plays a role in platelet cross-linking and platelet plug expansion [105]. Direct binding of VWF to the eggshell by the A1 domain may thus have two effects. VWF may be directly involved in the binding

of eggs to the endothelium by forming a bridge between the eggshell and the extracellular matrix. Indirectly, binding of VWF in combination with the binding of platelets [203] may induce platelet adhesion, platelet activation and secondary haemostasis, allowing the formation of a stable clot. In turn, this should facilitate a firm binding of the egg to the endothelium. Next to induction of clot formation, the schistosome egg also contains fibrinolytic capacity, as it has been shown that soluble extracts of eggs contain a plasmin-like fibrinolytic enzyme activity that could counteract uncontrolled thrombogenesis [51].

Coagulation normally occurs in the case of bleeding, when endothelium is damaged and extracellular matrix is exposed to which VWF can bind. Damage to the endothelium is in itself an activator of platelets and blood coagulation, as are shear stress and turbulence [177]. In case of schistosomiasis, shear stress can be caused by the presence of the relatively large worms and eggs. Attachment of adult worms to the endothelium may cause endothelial damage that leads to activation of endothelial cells [168]. As coagulation is initiated on eggshells in the near presence of damaged and activated endothelium, VWF bound to the egg may anchor the eggshell to the endothelium, after which platelet aggregation and fibrin clot formation can stabilise the binding.

Extravasation is the first step of excretion and is likely to occur rapidly, before eggs have fully matured and start secreting enzymes and other material. *In vitro* adhesion of eggs to endothelium takes place within 2 h [98, 126]. Within 4 h after adhesion to the vascular endothelium, endothelial cells actively migrate over the eggs [64]. This way the eggs are removed from the blood vessel. The binding site by which VWF binds to *S. mansoni* eggs is not easy to resolve experimentally, as this target cannot be isolated because the eggshell is composed of a heavily cross-linked protein structure [48]. Furthermore, VWF is a very large protein that has no catalytic activity and its primary function is binding to other proteins via protein-protein interactions. These interactions do not resemble classical receptor-ligand interactions, but are rather complex protein-protein interactions with induced conformational changes. In addition, VWF comprises many different domains for protein-protein interactions and even domain A1, which is crucial for binding to schistosome eggs, binds to a large variety of structures, such as glycoprotein Ib (GPIb), *Staphylococcus* protein A, Snake Venom Metalloproteinase, collagen and heparin [156]. For these reasons, the molecular mechanisms by which VWF binds to most other well-known interacting partners has not yet been resolved, and this prohibits the identification of potential VWF binding sites in the schistosome

eggshell on the basis of structural homology to a known target.

In conclusion, our data demonstrated that eggshells of *S. mansoni* bind a specific set of plasma proteins, including VWF. We suggest that binding of plasma proteins to eggshells is physiologically relevant and a crucial step in extravasation as it promotes binding to damaged or activated endothelium. It thereby prevents eggs being swept into the circulation and it facilitates egg extravasation from the blood vessel, which is the first step in excretion of the egg.

Acknowledgements

We thank Irina Dragan (Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands) for expert technical support.

Supplementary data

Table S1: List of proteins identified in human plasma versus proteins of human plasma bound to *Schistosoma mansoni* eggshells. The band numbers (BN) refer to the gel slices cut from SDS-PAGE as shown in Fig. 4.2. For each gel slice all protein identifications with at least four unique peptides are listed. PC, protein coverage; TP, total number of peptides; UP, number of unique peptides.

BN	Plasma			Eggs1			Eggs2					
	Protein name	PC	TP	UP	Protein name	PC	TP	UP	Protein name	PC	TP	UP
1	Albumin	21.8	19	14	Fibronectin 1	20.5	47	29	Fibronectin 1	32.0	112	56
1	Inter-alpha (Globulin) inhibitor H2	4.3	5	4					Fibrinogen alpha chain	6.9	6	6
1									Collagen, type II, alpha 1	4.8	6	5
2	Serum albumin	25.3	22	14	Fibronectin 1	38.4	160	66	Fibronectin 1	30.9	117	51
2	Alpha-2-macroglobulin	8.0	11	11	Talin 1	5.5	9	8	Talin 1	3.4	5	5
2	Fibronectin 1	7.1	13	11								
3	Serum albumin	36.5	36	21	Fibronectin 1	11.8	26	20	Fibronectin 1	15.2	42	30
3	Alpha-2-macroglobulin	15.8	25	22	Collagen, type II, alpha 1	2.9	6	4	Collagen, type II, alpha 1	2.9	4	4
3	Ceruloplasmin (ferroxidase)	11.5	11	10					Talin 1	1.5	4	4
3	Pregnancy zone protein	8.4	6	6								
3	Complement C3	3.7	5	4								
4					Fibronectin 1	2.8	7	5	Fibronectin 1	4.9	11	8
4									Fibrinogen alpha chain	4.7	4	4
5	Serum albumin	49.6	64	29								
5	Serotransferrin	33.4	26	21								
5	Ig mu chain C region	18.1	8	7								
5	Complement C3	4.9	5	5								
6	Serum albumin	75.9	132	46	Fibrinogen alpha chain	30.6	49	28	Serum albumin	32.8	21	16
6	Fibrinogen alpha chain	22.5	15	14	Albumin (Fragment)	22.7	6	5	Fibrinogen alpha chain	30.9	65	31
6	Uncharacterized protein	12.8	8	7	Vitronectin	19.7	14	9	Vitronectin	16.7	14	8
6					Fibronectin 1	3.9	5	5	Fibronectin 1	4.9	9	8
6									Collagen, type II, alpha 1	3.5	5	4
7	Alpha-1-antitrypsin	51.2	28	17	Fibrinogen beta chain	41.1	33	22	Fibrinogen beta chain	35.6	27	19
7	Serum albumin	40.1	42	21	Fibrinogen gamma chain	30.2	17	12	Uncharacterized protein	19.5	8	7
7	Ig gamma-1 chain C region	36.1	14	10	Fibrinogen alpha chain	27.0	27	22	Fibrinogen alpha chain	19.2	19	16
7	Fibrinogen beta chain	22.8	14	10	Albumin	20.7	14	9	Fibrinogen gamma chain	16.5	8	6
7	Ig alpha-1 chain C region	15.6	6	4	Vitronectin	16.7	7	6	Fibronectin 1	5.1	6	6
7					Fibronectin 1	5.8	9	9				

Table S1 continued

BN	Plasma Protein name	PC	TP	UP	Eggs1 Protein name	PC	TP	UP	Eggs2 Protein name	PC	TP	UP
8					Fibrinogen gamma chain	42.7	34	17	Fibrinogen gamma chain	34.7	30	13
8					Fibrinogen alpha chain	23.2	20	16	Actin, beta	19.1	7	7
8					Actin, beta	19.1	6	6	Fibrinogen alpha chain	16.7	16	12
8					Fibrinogen beta chain	13.2	4	4	Fibrinogen beta chain	16.5	6	6
8					Albumin	12.5	9	7	Uncharacterized protein	10.0	7	6
8					Fibronectin 1	9.7	18	16				
9	Serum albumin	25.1	19	13	Actin, beta	37.0	31	19	Fibronectin 1	7.0	12	9
9					Apolipoprotein A-IV	21.7	10	8	Actin, beta	32.2	22	17
9					Fibrinogen alpha chain	21.7	14	12	Apolipoprotein A-IV	22.7	13	9
9					Fibronectin 1	6.5	12	10	Fibrinogen alpha chain	9.8	5	5
9								Fibronectin 1	2.9	7	6	
10	Serum albumin	26.6	25	14	Fibrinogen alpha chain	4.2	8	4	Fibrinogen gamma chain	10.2	11	6
10	Haptoglobin	19.4	16	8				Clusterin	9.8	13	7	
10	Apolipoprotein A-IV	10.1	4	4				Fibrinogen alpha chain	8.5	11	6	
10	Fibrinogen alpha chain	5.5	7	5				Fibronectin 1	2.8	6	6	
10	Complement C3	2.3	4	4								
11	Albumin	10.2	8	5	Apolipoprotein E	24.3	10	7	Apolipoprotein E	26.8	9	8
11					Clusterin	15.4	17	8	Clusterin	16.7	12	9
11					Fibrinogen alpha chain	9.7	10	7	Fibrinogen alpha chain	11.0	8	6
11					Fibronectin 1	3.0	7	6	Fibrinogen gamma chain	10.2	8	5
12	Apolipoprotein E	19.9	6	6	Apolipoprotein E	42.3	36	15	Apolipoprotein E	34.7	31	13
12	Serum albumin	18.7	9	8	Fibrinogen alpha chain	11.9	7	5	Fibrinogen alpha chain	10.7	12	8
12	Fibrinogen alpha chain	6.1	6	4	Fibrinogen gamma chain	10.2	9	6	Fibrinogen gamma chain	10.6	10	6
12					Fibronectin 1	3.4	12	8				
13	Uncharacterized protein	17.2	12	8					Fibronectin 1	3.0	5	5
14	Albumin	19.9	11	10	Actin, beta	12.3	4	4	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Fragment)	22.8	5	4
15	Apolipoprotein A-I	24.1	11	4								

Chapter 5

Evaluation of molecular and serological methods for diagnosing two cases of neuroschistosomiasis

Saskia deWalick¹

Rob Koelewijn²

Aloysius G.M. Tielens¹

Jaap J. van Hellemond^{1,2}

Manuscript in preparation

¹ Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands

² Laboratory of Parasitology, Harbour Hospital and Institute for Tropical Diseases, Rotterdam, The Netherlands

Abstract

Neuroschistosomiasis is the most severe ectopic form of schistosomiasis. To prevent severe neurological damage and permanent disability, rapid diagnosis and treatment are crucial. However, diagnostic tools for rapid diagnosis of neuroschistosomiasis are currently lacking. Here we present two cases of confirmed schistosome myelopathy and compared molecular as well as distinct serological methods for their usefulness to diagnose neuroschistosomiasis. Real-time quantitative PCR and Western blots did not provide positive test results for our patients, but quantification of specific antibody levels against adult worm antigens and egg antigens in serum and cerebrospinal fluid was indicative for intrathecal antibody production directed against schistosome eggs. Hence the proposed egg/worm antibody index can be a valuable tool to distinguish between on the one hand patients with true neuroschistosomiasis and on the other hand patients that have systemic schistosomiasis but no eggs in the spinal cord, where the transverse myelitis is caused by a different disorder.

5.1 Introduction

Schistosomiasis is a tropical disease affecting an estimated 200 million people worldwide, mainly in sub-Saharan Africa [74]. The disease is caused by a parasitic flatworm of the *Schistosoma* genus. Eggs laid by the female parasite that are not excreted accumulate in host tissue and cause the vast majority of the disease symptoms. Adult worms usually reside in the mesenteric veins or in the blood vessels surrounding the urine bladder. Sometimes, however, worm pairs reside in ectopic locations. The most severe ectopic form of schistosomiasis is transverse myelitis. Transverse myelitis is an inflammation of the spinal cord causing neurological dysfunction such as muscle weakness, sensory loss, bladder dysfunction and finally paraplegia. In case of neuroschistosomiasis, it is caused by the inflammatory response of the host to the schistosome eggs that are trapped in the spinal cord. The eggs are deposited *in situ* after aberrant migration of adult worms to the central nervous system (CNS), or by egg dissemination via the bloodstream through retrograde venous flow into the Batson vertebral epidural plexus, which connects the deep iliac veins and inferior vena cava with veins of the spinal cord [26, 27, 154]. The inflammatory lesions around the eggs suppress the spinal cord causing neurological dysfunction. Neuroschistosomiasis has an acute or subacute onset and may then progress to full paralysis within 15 days if untreated [63]. Diagnosis of neuroschistosomiasis is difficult and the disorder is often not recognized and therefore probably underdiagnosed [62]. True prevalence is unknown, but a study in Brazil showed that 5.6% of non-traumatic and non-tumoural myelopathies were caused by neuroschistosomiasis [29].

Normally, the cerebrospinal fluid (CSF) contains little protein and few cells. During inflammatory diseases of the CNS, the blood-brain-barrier is usually damaged at least to some extent. Therefore, leakage of antibodies from blood plasma into the CSF is likely to occur. Hence, the presence of anti-schistosome antibodies in the CSF could either be the result of intrathecal antibody production due to neuroschistosomiasis or it could be the result of leakage of antibodies from the plasma into the CSF due to damage to the blood-brain-barrier caused by a distinct neurological disorder. Leakage of plasma antibodies into the CSF is a-specific and the profile of the antibodies leaking will resemble that of plasma antibodies. Intrathecal antibody production on the other hand is specific and the antibodies produced are directed against the antigens of the pathogen in the central nervous system. As the number of (migrated) B-cells in the CSF is limited, the different

antibodies that are produced intrathecally are also limited. In case of neuroschistosomiasis, where the immune response is directed against the schistosome eggs that have crossed the blood brain barrier, intrathecal antibody production is expected to be differentially directed to eggs more than to worms. However, it should be realized that the egg and the adult worm share many of their antigenic proteins [40].

As therapeutic delay may quickly lead to severe neurological damage and permanent disability, rapid diagnostic tools are needed [28]. Diagnosis of spinal cord neuroschistosomiasis usually relies on the clinical diagnosis of transverse myelitis by imaging techniques (such as MRI) to show edema and swelling of the spinal cord, in combination with diagnosis of an active infection with *Schistosoma* spp. and exclusion of other causes of myelitis. Current methods cannot properly discriminate between neuroschistosomiasis as the cause of the transverse myelitis and transverse myelitis with other causes in patients with systemic schistosomiasis. The gold standard for diagnosis of neuroschistosomiasis is retrieval of eggs from the spinal cord [28]. However, this invasive method is rarely used, because of the risk for serious complications. Therefore, all current methods of diagnosis are non-specific for neuroschistosomiasis and are based on exclusion of other causes of the myelopathy. It is important to exclude any other infectious cause of myelopathy and to be certain of the diagnosis neuroschistosomiasis because the therapy for neuroschistosomiasis includes a high dose of corticosteroids that will have adverse effects in patients with transverse myelitis caused by viral or bacterial pathogens [183].

Here we report two cases of schistosome myelopathy and examined the usefulness of three methods to diagnose neuroschistosomiasis. These methods have proven to be useful in the diagnosis of other infections in the CNS such as neurosyphilis, toxoplasmosis, and varicella zoster virus infection. The following tests were examined: (1) detection of schistosome DNA in CSF by real-time semi-quantitative PCR in order to demonstrate the presence of the parasite in the CNS, (2) Western blot analysis to demonstrate a qualitatively different anti-schistosome antibody composition in CSF compared to serum, and (3) indirect hemagglutination assay (IHA) and ELISA to demonstrate a quantitatively different anti-schistosome antibody composition in CSF compared to serum, a method previously described [42].

5.2 Materials and Methods

Case descriptions

Two patients (A and B) presented at the Erasmus University Medical Center with the clinical symptoms of progressive transverse myelitis. Paired serum and CSF samples from each patient were taken at presentation and once thereafter.

Patient A was a 35 year-old male who in May 2008 presented to the emergency department with progressive pain and muscle weakness in the upper legs [42]. Two months prior to presentation, he suffered from diarrhea during a visit to Brazil. The pain in his legs started four days prior to hospital presentation and was accompanied by dysuria and a feeling of incomplete voiding. During the next few days, muscle weakness, sensory disturbances and bladder dysfunction increased. An MRI scan showed swelling and edema of the low-thoracic and lumbar spinal cord without abnormalities in the brain. Antibodies against schistosome antigens were found in serum and CSF and levels were increased after 11 days (Table 5.1). In a rectal biopsy one dead *S. mansoni* egg embedded in a granuloma was found. Based on these results and the negative test results for other infectious causes of transverse myelitis, the diagnosis neuroschistosomiasis was made and treatment was started. The patient received praziquantel (2 daily doses of 30 mg/kg for 3 days) and high dose of intravenous (i.v.) methylprednisolone (2 daily doses of 500 mg for 5 days) followed by oral prednisone (3 daily doses of 0.5 mg/kg) tapered gradually over the next several months. Within a week the patient's condition improved dramatically and he was discharged from the hospital. During the next months, muscle strength and sensibility improved, and bladder function normalized.

Patient B was a 59-year old male who presented in September 2010 at our neurology department. The patient was born in Suriname, a country to which he traveled regularly and his last visit was in 2009. In January 2010 he developed progressive lumbar pain irradiating to the lower limbs, with asymmetric loss of sensibility and progressive muscle weakness in the legs. The patient was able to walk for 15 to 30 minutes, but fell regularly. He had problems walking stairs and needed to pull himself on the handrail. The patient was incontinent for urine when in high need. Otherwise miction and defecation were not affected. MRI showed a lesion in the spinal cord at the low-thoracic and lumbar typical for myelitis transversa. The protein level (1.12 g/L) and the numbers of leukocytes ($19 \times 10^6/L$) and monocytes ($13 \times 10^6/L$) were elevated in CSF. Furthermore, antibodies against

schistosomes were found in paired serum and CSF samples, collected at presentation and 8 days later (Table 5.1). No eggs of schistosomes could be demonstrated by microscopic methods in feces or urine. Since no other cause for the transverse myelitis was found, the patient was treated with praziquantel (2 daily doses of 30 mg/kg for 3 days) and methylprednisolone (once daily 500 mg i.v. for 5 days) followed by oral prednisone (2 daily doses of 0.5 mg/kg tapered gradually over the next several months). The physical condition of this chronic patient improved significantly within a week, but the patient did not fully recover probably due to irreversible nerve damage caused in preceding nine months of disease.

DNA isolation and real-time PCR

A real-time PCR was performed on DNA extracted from 0.2 ml CSF using easyMag (bioMérieux, France) according to the manufacturer's instructions. Phocine Herpes Virus (PhHV) was added to the lysis buffer as an internal control. The used primers and probes have been designed based on sequences from *S. haematobium* (GenBank accession DQ677661), *S. mansoni* (AF503487) and *S. intercalatum* (U22166) [127]. We used the following PCR cycle conditions: a denaturation step of 15 min at 95 °C, followed by 50 cycles of 15s denaturation at 95 °C, 30s annealing at 60 °C and 30s elongation at 72 °C. Fluorescence was measured at each annealing step.

Western blot analysis

Worms and eggs were retrieved from experimentally infected hamsters. Worms and eggs were homogenized by sonification for 3 × 30 sec in phosphate buffered saline (PBS). The homogenate was spun down for 10 min at 18000 g. The obtained supernatant was used to load SDS-PAGE gels (5 µg adult worm protein or 10 µg egg protein, as measured by a Lowry protein assay using bovine serum albumin as a standard). Gels were blotted on PVDF membranes, blocked for 1 h in low fat milk powder in TBS-tween and subsequently incubated with either serum (1:1000) or CSF (1:100). The secondary antibody was HRP conjugated anti-human IgG (1:10000) (Dako, Glostrup, Denmark). Bands were visualized using ECL (Pierce, Rockford, IL, USA) on Hyperfilm (Amersham, GE healthcare, Diegem, Belgium).

Serological methods

Total albumin and total IgG were determined in serum and CSF by routine clinical chemistry methods. Antibody titers to schistosome worm-antigens were determined using a commercial IHA (Fumouze Diagnostics, Levallois-Perret, France) and agglutination at serum titers of 1:80 and over are positive for schistosomiasis [189]. Schistosome egg-specific antibodies were determined by ELISA of soluble worm antigen (SEA) as described before [189]. Anti-egg antibody levels were expressed as Arbitrary Units (AU) calculated as Optical Density (OD) \times dilution / 10 and AU values >88 in serum are positive test results for schistosomiasis.

Theory and calculation of serological indices

The albumin index is a measure for the integrity of the blood-brain barrier and values ≥ 0.0090 indicate an impaired function of the blood-brain barrier [181].

$$\text{Albumin index} = \frac{AC}{AS}$$

AC = albumin concentration in CSF (g/L)

AS = albumin concentration in serum (g/L)

The total IgG index is a measure for increased concentrations of total IgG in CSF and values ≥ 0.70 are already suggestive for intrathecal antibody production, since albumin leaks more easily through the blood-brain barrier when compared to antibodies [60, 104, 142].

$$\text{Total IgG index} = \frac{IC/AC}{IS/AS}$$

IC = total IgG CSF (g/L)

IS = total IgG serum (g/L)

Intrathecal antibody production will occur upon any inflammatory response in the CNS, and therefore, the total IgG index is not specific for schistosomiasis. Demonstration of schistosome-specific antibodies in CSF is thus more informative and this has indeed been shown to be an indication for neuroschistosomiasis [61, 131]. However, the presence of schistosomes-specific antibodies in CSF does not discriminate intrathecal production of these antibodies from antibody leakage from the plasma into the CSF. Two methods have been developed to correct for leakage of antibodies from plasma into the CSF. Antibody titers can be corrected

for albumin concentrations in CSF, which is not commonly used, or corrected for total IgG in CSF, which is known as the specific antibody index. The cut-off value for specific antibody indices is 1.5, with higher levels indicative for intrathecal production of specific antibodies [143].

$$\text{Schistosome-specific antibody index} = \frac{EC/IC}{ES/IS}$$

EC = anti-egg IgG in CSF (OD × dilution)

ES = anti-egg IgG in serum (OD × dilution)

Transverse myelitis in neuroschistosomiasis is caused by the immune response directed to schistosome eggs. To differentiate between leakage of plasma antibodies and intrathecal antibody production, we also used an index which calculates whether the anti-schistosome IgG response in CSF is more egg-directed than in serum. We used the egg–worm antibody index as a measure for the relative enrichment of anti-egg IgG over anti-worm IgG in CSF when compared to serum.

$$\text{Egg - worm antibody index} = \frac{EC/WC}{ES/WS}$$

WC = anti-worm IgG in CSF (titer)

WS = anti-worm IgG in serum (titer)

This is the most specific antibody index which will only show increased values when antibodies directed against eggs of schistosomes are produced intrathecally, which only occurs when neuroschistosomiasis is the cause of the transverse myelitis. Following Luger *et al.* (2000)[104], we suggest a cut-off value of 2 for this egg–worm antibody index, representing a two-fold increase in egg-specific antibodies in CSF. This means that values ≥ 2 indicate intrathecal production of IgG specific for schistosome eggs, and thus indicate neuroschistosomiasis.

5.3 Results

Real-time semi-quantitative PCR

Real-time semi-quantitative PCR is a very sensitive method to detect specific DNA and it has been used to detect schistosome infections in blood [36, 198, 204]. We hypothesized that if the transverse myelitis was caused by eggs of *Schistosoma* species, some of their DNA might leak into the CSF and this schistosome DNA

Table 5.1: Serological data of paired serum-CSF samples. The results are shown of serological analysis of the two paired serum and CSF samples collected from two patients (A and B) at presentation (day 0) and at day 11 and 8 after presentation respectively. Total albumin and total IgG were determined by routine clinical chemistry methods. Antibody titers to schistosome worm antigen were determined by a commercial indirect hemagglutination assay (IHA) and antibody levels of schistosome egg-specific antibodies were determined by ELISA and expressed as Arbitrary Units (AU) calculated as Optical Density (OD) \times dilution / 10.

Sample	Type	Albumin (g/L)	Total IgG (g/L)	Worm IHA titer	SEA ELISA AU
A0	serum	43.3	12.4	2560	229.6
	CSF	0.859	0.128	16	3.95
A11	serum	41.2	9.8	5120	286.8
	CSF	0.771	0.228	32	7.73
B0	serum	44.0	13.3	5120	136.0
	CSF	0.803	0.169	4	1.88
B8	serum	46.7	13.7	5120	185.2
	CSF	0.438	0.070	4	1.63

can then be detected by a sensitive PCR method. Hence, for our two patient cases we examined the usefulness of real-time semi-quantitative PCR on CSF for the diagnosis of neuroschistosomiasis. The used PCR method targets the internal-transcribed-spacer-2 (ITS2), a multi-copy sequence in schistosomes. This assay had a detection limit of 0.1 pg of schistosome DNA per assay as determined in our laboratory by the use of dilution series of purified chromosomal DNA of adult schistosomes (not shown). Thus, the detection limit is less than the amount of DNA present in one single schistosome cell. Despite the high sensitivity of the PCR, schistosome DNA could not be amplified in any of the CSF samples of our two neuroschistosomiasis patients while internal controls were amplified properly. Therefore, detection of schistosomal DNA by a sensitive PCR method does not seem to be useful for diagnosing neuroschistosomiasis.

Serology

When intrathecal antibody production against schistosome antigens can be demonstrated, neuroschistosomiasis is confirmed. Therefore, it is important to distinguish between leakage of antibodies from blood plasma into the CSF and intrathecal antibody production. In the case of leakage of antibodies against schistosomes

from the plasma into the CSF, the cause of the transverse myelitis is not necessarily neuroschistosomiasis but could be anything that causes damage to the blood-brain-barrier in patients that are coincidentally infected with *Schistosoma* species. Intrathecal antibody production is characterized by a raised total IgG index and/or oligoclonal bands in the CSF. Serological diagnosis of neuroschistosomiasis can only be made when intrathecal production of specific antibodies against schistosomes can be demonstrated. We performed Western blots, IHA and ELISA to qualify and quantify the anti-schistosome response in both serum and CSF of two patients with neuroschistosomiasis to examine their usefulness for the diagnosis of neuroschistosomiasis. Western blots using worm and egg fractions were performed with both serum and CSF to detect intrathecal production of schistosome-specific antibody production as prove for neuroschistosomiasis (Fig. 5.1). Many protein bands were detected by Western blot using serum and CSF of neuroschistosomiasis patients (Fig. 5.1A and B). However, protein bands were also detected when blots were incubated with serum and CSF from patients infected with other pathogens (Fig. 5.1C and 1D), demonstrating that not all reacting protein bands are the result of schistosomes-specific antibodies. Furthermore, the banding pattern to worm and egg fractions was similar in CSF and serum from the same neuroschistosomiasis patient (Fig. 5.1A and B). In CSF of patient B the Western blot with egg antigens, shows a band at 35 kD that seems to be absent in serum, but interpretation is difficult due to the reduced staining reactivity of the serum blot compared to the CSF blot (Fig. 5.1B). Therefore, the Western blots could not reveal an obvious differential schistosome-specific antibody response in CSF versus serum in our neuroschistosomiasis patients. Hence, no schistosome-specific intrathecal antibody production could be demonstrated. These results are consistent with the findings of Pammenter *et al.* (1991)[131], and therefore, our results support their conclusion that there is no additional diagnostic value for Western blot over conventional ELISA in the diagnosis of neuroschistosomiasis. We used an anti-egg ELISA and a worm IHA to quantify the antibody responses in both CSF and serum to native schistosome antigens. Albumin and total IgG were also measured (Table 5.1). With these measurements we were able to calculate different indices as mentioned above which are informative about the amount of damage to the blood-brain barrier, the amount of intrathecal antibody production and whether the intrathecal IgG response directed against schistosomes is egg-skewed or adult worm-skewed. In all our samples the albumin index was elevated, indicating a damaged blood brain barrier (Table 5.2). This further supports the

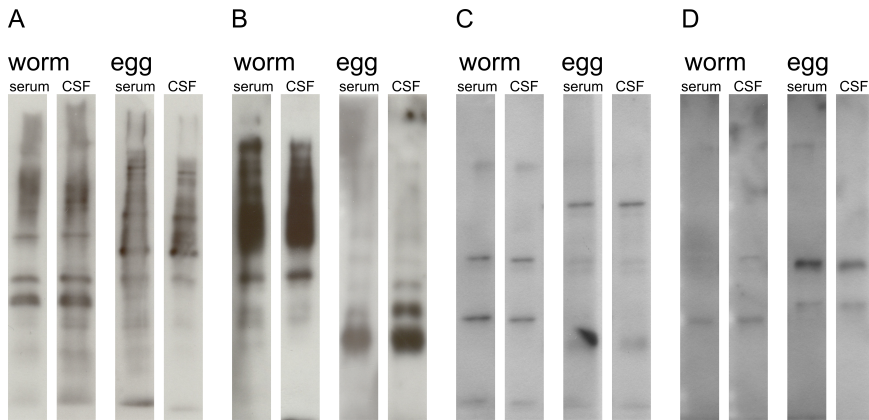


Figure 5.1: Western blots on worm and egg antigen of paired serum and CSF samples. Soluble worm and egg fractions were blotted on PVDF membranes and incubated with serum (1:1000) or CSF (1:100) of patient A (A) and patient B (B), a patient with bacterial meningitis (C) and a patient with cerebral toxoplasmosis (D).

clinical picture of transverse myelitis but does not give any clues towards the cause of the transverse myelitis. Where the albumin index is a measure for blood-brain damage, the total IgG index can indicate intrathecal antibody production. An elevated total IgG index in schistosome-infected patients is indicative for neuroschistosomiasis [60], but is not specific for neuroschistosomiasis and may occur in any infectious cause of transverse myelopathy. Only one of each of our paired serum-CSF samples of the two patients had an increased total IgG index indicating an infectious cause of the transverse myelitis (Table 5.2). Hence, this index does not seem to be a sensitive method for the diagnosis of transverse myelitis caused by infectious agents.

Pammenter *et al.* (1991)[131] and Ferrari *et al.* (1995)[61] showed that the presence of schistosome-specific IgG in CSF is indicative for neuroschistosomiasis. However, this test has a low negative predictive value [131] or lacks sensitivity at the suggested cut off of 1.4 $\mu\text{g/ml}$ [61]. Furthermore, this test does not discriminate between antibody leakage from plasma and intrathecal antibody production. A schistosome-specific antibody index or the egg-worm antibody index can confirm intrathecal egg-specific antibody production more specifically than a total IgG index or the absolute levels of anti-worm or anti-egg IgG in the CSF. Magalhães-Santos *et al.* (2003)[107] noted that the IgG1 isotype levels were higher in the CSF than in serum of patients with neuroschistosomiasis. This suggests local

Table 5.2: Calculated indices of paired serum-CSF samples. The four calculated indices for the paired serum-CSF samples. The albumin index [albumin CSF (g/L)/albumin blood (g/L)] as a measure for the integrity of the blood-brain barrier, the total IgG index [(total IgG CSF (mg/L)/albumin CSF (g/L))/(total IgG serum (mg/L)/albumin serum (g/L))] as a measure for concentrations of total IgG in CSF, the schistosome-specific antibody index [(anti-egg IgG in CSF (OD × dilution)/total IgG in CSF)/(anti-egg IgG in serum (OD × dilution)/total IgG in serum)] as a measure for enrichment of egg-specific IgG compared to total IgG and the egg-worm antibody index [(anti-egg IgG in CSF (OD × dilution)/anti-worm IgG in CSF (titer))/(anti-egg IgG in serum (OD × dilution)/anti-worm IgG in serum (titer))] as a measure for the relative enrichment of anti-egg IgG over anti-worm IgG in CSF when compared to serum.

Sample	Albumin index	Total IgG index	Schistosome	
			specific antibody index	Egg-worm antibody index
A0	0.020	0.52	1.7	2.75
A11	0.019	1.24	1.2	4.31
B0	0.018	0.70	1.1	17.65
B8	0.009	0.55	1.7	11.27

synthesis of IgG1. However, they did not calculate the specific IgG1 antibody index to test for specific intrathecal antibody production. Two of our four samples tested had a positive schistosome-specific antibody index - one from each patient - showing that this antibody index is more sensitive than the total IgG index, but still not sensitive enough for the diagnosis of neuroschistosomiasis. Thus, in our patients, the schistosome-specific antibody index (not divided by subclasses) was not sensitive enough to diagnose neuroschistosomiasis as the cause of transverse myelitis. However, the egg-worm antibody index did indicate intrathecal antibody production in all paired serum-CSF samples (Table 5.2), thereby confirming neuroschistosomiasis. Both our patients had increased indices. Patient A had a slightly positive index for the first paired serum-CSF sample which had increased to 4.31 after 11 days. Patient B had highly elevated indices at both time points.

5.4 Discussion

Although PCR detection of viral and bacterial pathogens can be used to diagnose several infectious diseases in the CNS, this technique was not capable of detecting schistosome DNA in the CSF of confirmed neuroschistosomiasis patients. Apparently, insufficient amounts of DNA of the eggs are present in the CSF. Viral and bacterial pathogens infecting the CNS replicate and in general disseminate,

thereby multiplying their amount of DNA in spreading areas of the CNS. These living micro-organisms or DNA fragments of them can thus be retrieved in the CSF sample collected by puncture. However, schistosomes nor their eggs multiply within the host. Thus, the amount of schistosome DNA from the eggs in the CSF is relatively restricted. Furthermore, the eggs will be encapsulated in granulomas formed by the inflammatory immune response that is elicited upon protein excretion by the developing miracidium inside the egg, prohibiting extensive diffusion of DNA into the CSF. We conclude that real-time semi-quantitative PCR has no additional value in the diagnosis of neuroschistosomiasis, although PCR on stool samples can be useful for confirmation of an active schistosome infection in addition to serology and traditional microscopy [180].

In addition to DNA amplification methods, we examined various serological methods for the usefulness to diagnose neuroschistosomiasis. Intrathecal antibody production against schistosomal eggs could not be demonstrated by Western blot techniques, but the schistosome-specific antibody index and especially the egg-worm antibody index were indicative for neuroschistosomiasis in our patient cases. A further advantage of the egg-worm antibody index is that the outcome is independent of the diagnostic method used. It can be calculated with the results of any two serological tests for the diagnosis of schistosomiasis based on egg and worm antigens. Therefore, this concept could also be useful to diagnose neuroschistosomiasis caused by *S. japonicum* and *S. mekongi*, the schistosome species present in Asia, by using serological methods detecting egg and worm antigens of these species [99, 101]. Every laboratory can implement a ratio with the diagnostic methods that are currently in use in that laboratory. In addition, the test is not biased by the presence of cross reacting antibodies in serum from previous infections, as a positive egg-worm antibody index can only be achieved by increased levels of antibodies in CSF due to intrathecal production. On the other hand, all four test results need to be positive in order to calculate the index, which might limit the sensitivity of this test in endemic areas as antibody titers against schistosomes tend to decrease in time during chronic schistosomiasis due to immune suppression induced by the schistosomes [193]. In conclusion, quantification of antibody levels against worm and egg antigens in CSF and serum to determine the egg-worm antibody index is a simple and relatively rapid method that seems to be useful in the diagnosis of neuroschistosomiasis. However, confirmation on a larger set of neuroschistosomiasis patients is required for the ultimate validation of this method.

Chapter 6

The tegumental surface membranes of *Schistosoma mansoni* are enriched in parasite-specific phospholipid species

Kim Retra^{1,2*}

Saskia deWalick^{3*}

Marion Schmitz¹

Maria Yazdanbakhsh²

Aloysius G.M. Tielens^{1,3}

Jos F.H.M. Brouwers¹

Jaap J. van Hellemond³

*These authors contributed equally to this work

International Journal for Parasitology 2015.

Accepted for publication.

¹ Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

² Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands

³ Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands

Abstract

The complex surface structure of adult *Schistosoma mansoni*, the tegument, is essential for survival of the parasite and consists of a syncytium of fused cells covered by two closely-apposed lipid bilayers that form the interactive surface with the host. In order to identify parasite-specific phospholipids present in the tegument, the species compositions of the major glycerophospholipid classes, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI), including lysophospholipid species were analysed in adult *S. mansoni* worms, in isolated tegumental membranes and in hamster blood cells. It was shown that there are large differences in species composition in all four phospholipid classes between the membranes of *S. mansoni* and those of the host blood cells. The species compositions of PS and PC were strikingly different in the tegument compared to the whole worm. The tegumental membranes are especially enriched in lysophospholipids, predominantly eicosenoic acid (20:1)-containing lyso-PS and lyso-PE species. Furthermore, the tegument was strongly enriched in PC that contained 5-octadecenoic acid, an unusual fatty acid that is not present in the host. As we showed earlier that lysophospholipids from schistosomes affect the host-parasite interaction, excretion of these tegument-specific phospholipid species was examined *in vitro* and *in vivo*. Our experiments demonstrated that these lysophospholipids are not significantly secreted during *in vitro* incubations and are not detectable in peripheral blood of infected hosts. However, these analyses demonstrated a substantial decrease in PI content in blood plasma of schistosome infected hamsters, which might indicate that schistosomes influence exosome formation by the host.

6.1 Introduction

Schistosoma mansoni is a parasitic worm that causes schistosomiasis, a disease that afflicts over 200 million people and numerous animals in many rural areas in tropical countries [74]. Adult *S. mansoni* worms are able to maintain themselves for many years in the blood vessels of their mammalian hosts [75]. Despite their relatively large size (1 cm long with a diameter of 0.5 mm) compared to veins, and their abundant exposure to immune cells present in the blood of the host, the parasite apparently prevents clot formation and an adequate immune response of its host [108, 115, 137]. Although the underlying molecular mechanisms involved in long-term parasite survival are not yet completely understood, the tegument surface structure of schistosomes is of crucial importance for parasite survival and modulation of the host response [79, 171].

The tegument of flatworms is a unique structure in nature that forms the interactive surface with the host. The tegument of schistosomes consists of a syncytium of fused cells covered by two closely-apposed lipid bilayers, constituting an inner plasma membrane and an outer membranocalyx [23, 113, 171]. Multiple studies characterized the protein composition of these tegumental surface membranes, which demonstrated that these membranes contain a specific set of proteins comprising nutrient transporters, structural membrane proteins, several proteins derived from the host and many proteins with a yet unknown function [15, 31, 184]. The tegument of *S. mansoni* is also enriched in proteins that share no sequence similarity to any sequence present in databases of species other than schistosomes, demonstrating that the unique tegumental structures comprise multiple unique proteins that are likely to fulfil schistosome-specific functions involved in parasite survival [15, 171, 184].

Next to proteins, the membranes of the tegument contain many distinct lipids and it was shown that eicosenoic acid (20:1), a rare species in the mammalian host, is a major component of the phospholipids of the tegumental membrane [2]. The few early studies on the lipid compositions of the tegumental membranes provided little information on species compositions and were restricted by the limited technical possibilities of those days [2, 151]. More recent studies on tegumental membranes of schistosomes using MS techniques showed that the species composition of the phospholipids in the tegumental membranes differ not only substantially from those of blood cells of the host but also from those of whole adult worms [21, 190]. So far, the diacyl-phospholipid species

composition of the tegument has been characterized of the two most abundant phospholipid classes in membranes of schistosomes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [21]. However, the species compositions of the less abundant phospholipid classes, such as phosphatidylserine (PS) and phosphatidylinositol (PI) have not yet been characterized in the tegument. These phospholipids are of interest as they are known to function as precursors for the production of several potent signalling molecules. Furthermore, schistosome lyso-PS (monoacylphosphatidylserine) species have been shown to activate Toll-Like Receptor 2 (TLR2) and affect dendritic cells of the host in such a manner that mature dendritic cells induce the development of IL-10 producing regulatory T cells, a process known to result in down regulation of the immune response [187]. The present study provides a comprehensive analysis of the species compositions of all major glycerophospholipid classes, including PS, PI and lysophospholipids in isolated membranes of whole adult *S. mansoni* worms, in isolated tegumental membranes, and in membranes of host blood cells. In order to identify parasite-specific lipids the phospholipid species compositions in whole adult worms were compared to those of host blood cells. Furthermore, to identify phospholipid species enriched in the tegumental membranes, the phospholipid species compositions of tegumental membranes were compared to those of whole adult worms. Finally, the possible excretion into the environment of schistosome-specific phospholipids enriched in the tegumental membranes was investigated both *in vitro* and in infected hamsters.

6.2 Materials and Methods

Chemicals and materials

All solvents used were from Lab-Scan (Dublin, Ireland) and were of HPLC grade. Ammonium acetate, serine and Silica-60 for column chromatography were purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Baker (St. Louis, MO, USA) and CM-52 was obtained from Serva (Heidelberg, Germany).

Isolation of tegument, membranes of host blood cells and lipids in host blood

Adult *S. mansoni* parasites were obtained by perfusion via the heart with 0.9% [w/v] NaCl of ether- or isofluorane-anesthetized hamsters at 45–49 days after infection. Isolated adult *S. mansoni* worms were washed in Hanks Balanced Salt Solution (HBSS) [161] before tegumental membranes were isolated by a freeze-thaw method according to Roberts *et al.* (1983)[147] as described by Brouwers *et al.* (1999)[19]. Briefly, adult worms in HBSS were plunged drop by drop into liquid nitrogen. After thawing on ice, worms were extensively washed with ice-cold Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 0.9% [w/v] NaCl, 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride). The tegument membrane complex was removed from the worms by applying 10 vortex pulses of 1 sec. The supernatant, containing the tegumental membranes, was passed over a fine stainless-steel mesh and the filtrate was centrifuged at 5000g for 30 min at 4 °C. The isolated tegument membrane pellets were frozen at -20 °C until further use.

Blood was drawn from anesthetized hamsters by heart punctures. To determine the phospholipid composition of host blood cells, blood from non-infected hamsters was diluted (1:5, v/v) in water in order to lyse the red blood cells by hypotonic shock, shortly before lipid extraction. To analyse the phospholipids possibly excreted by schistosomes while living in the blood of the host, blood plasma from infected hamsters was prepared by centrifugation for 10 min at 1200g of citrated blood collected 45–49 days after infection with *S. mansoni*. As a control, plasma of non-infected hamsters was obtained by the same procedure.

Incubation of adult worms and lipid extraction from culture supernatant and from hamster blood

Adult *S. mansoni* worms were isolated by perfusion with warm (37 °C) 0.9% [w/v] NaCl and were then incubated for 2 h in S100 medium, which contained 20 mM Hepes (pH 7.4), 85 mM NaCl, 5.4 mM KCl, 0.7 Na₂HPO₄, 1 mM MgSO₄, 1.5 mM CaCl₂, 25 mM NaHCO₃, 100mM glucose and 20% heat-inactivated human serum. After incubation the adult worms were removed from the medium and ice-cold tri-chloric acid (TCA) was added to the culture supernatant after which the proteins were allowed to precipitate on ice for 30 min. The culture supernatant was subsequently spun down at 18000g for 10 min. Lipids were extracted according to

the method of Bligh and Dyer (1959) [12].

Lipid extraction

Lipids were extracted from the biological samples according to the method of Bligh and Dyer (1959) [12], with the minor modification that 6M HCl (0.1% v/v) was added at the second chloroform wash to increase recovery of acidic phospholipids. Neutral lipids were removed from the extracted lipids by fractionation on a 3 ml silica column prepared from 0.063–0.200 mm Silica 60. Lipid extracts were dissolved in chloroform and loaded on top of the silica column. Subsequently, neutral lipids were eluted with acetone (4 volumes) after which phospholipids and free fatty acids were eluted with methanol (4 volumes). This latter fraction was dried under nitrogen and stored at -20 °C until HPLC-MS analysis of the phospholipids.

Quantification and identification of phospholipid species composition

Isolated phospholipid samples were analyzed by an HPLC-MS method as previously described [145]. In short, phospholipids were separated by HPLC, using a Synergi 4 µm MAX-RP 18A column (250 × 3 mm) (Phenomenex, CA, USA). Elution was performed within 55 minutes with a decreasing linear gradient of water in methanol/acetonitrile, with serine and ammonium acetate used as additives. Mass spectrometry was performed using electrospray ionization on a Sciex 4000QTRAP (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Samples were analyzed in a multiple reaction mode (MRM) or by neutral loss scanning or precursor scanning. Data analysis was performed according to the manufacturers' protocols with Markerview version 1.0 software (Sciex, Toronto, Canada).

6.3 Results

This study aimed to provide a comprehensive characterization of the glycerophospholipid and lysophospholipid species compositions of the tegumental membranes of adult *S. mansoni* worms, in order to identify parasite and tegument-specific phospholipids that might be involved in host-parasite interactions. We used a universal HPLC-mass spectrometry method for the identification of the molecular species of the four most abundant phospholipid classes (PC, PE, PS and

PI) in homogenates of whole adult schistosomes, in isolated tegumental membranes of adult schistosomes and in blood cells of the host. The applied method separates isobaric species of all phospholipid classes prior to identification and quantification by mass spectrometry and therefore, allows detection and relative quantification of phospholipid species including lysophospholipids [145].

Tegumental membranes were isolated by a well-established freeze-thaw method originally developed by Roberts *et al.* (1983) [147]. A modified version of this method has been used in our laboratory to characterize the proteome and the species compositions of the phospholipids PE and PC in the tegumental membranes of adult schistosomes [18, 19, 22, 184].

Results of the current analysis of the species compositions of the analysed phospholipids in membranes of whole schistosomes (Table 6.1 and Tables S1-S3), are in general in agreement with our earlier study and the most abundant species of PS, PE, PC and PI in schistosomes were again found to be PS 18:0/22:4, PE 18:0/22:4, PC 16:0/20:1 and PI 18:0/22:4 [145]. The present study did not focus on ether-linked PE species, because the positive ion mode now used is less suitable for detection of these PE phospholipids [23]. Therefore, this study could not confirm the previously observed abundant presence of ether-linked PE species in schistosome membranes nor the specific enrichment of ether-linked PE species in tegumental membranes, such as 16:0/20:1 plasmalogen-PE [21].

PS species analysis

The HPLC-elution pattern of PS species derived from tegumental membranes differed drastically from that of membranes of whole worms and from that of membranes from blood cells, as the tegumental membranes clearly contain much more lyso-PS and etherlipid-PS (Fig. 6.1). Identification and quantification of the PS species demonstrated that schistosome membranes comprise many distinct PS species hardly present in the membranes of blood cells, such as PS 18:0/22:4, PS 18:0/20:1 and several etherlipids (Table 6.1). PS species containing a fatty acid of 22 or more carbon atoms were abundantly present in schistosomal membranes when compared to membranes of blood cells, 57% versus 9% respectively (Table 6.1). On the other hand, species with 22 or more carbon atoms are much less present in the tegument when compared to membranes of whole worms and comprise only 28% of the PS species in the tegument (Table 6.1).

In order to identify the PS species most different in abundance between mem-

branes of adult worms, the tegument and blood cells, a discriminant analysis was performed. This statistical method identifies the components that differ most between groups of samples and thus contribute most to the distinction between these samples. This discriminant analysis identified lyso-PS 20:1 ($m/z = 550$), lyso-PS 22:4 ($m/z = 572$) and the PS species with an m/z ratio of 772, 800, 804, 830 and 832 as the PS species most abundant in the tegumental membranes (Fig. S1). At least 4 of these 5 PS species contain ether-linked acyl chains (Table 6.1). Therefore, the PS species composition in the tegumental membranes is specifically enriched in ether-linked PS species (Fig. 6.1), which is similar to the already reported enrichment of ether-linked PE species in the tegument [21].

PE species analysis

Analysis of the species compositions of PE showed striking differences between PE in blood cells and in lipids of schistosomes (Table S1). The three most abundant species in PE of hamster blood cells (18:1/20:4, 18:1/18:2 and 16:0/18:1) are not even among the top 10 of the most abundant PE species in worms. The reverse is also true: the most abundant PE species in schistosomes (18:0/22:4, 18%) ranks as number 15 in PE species of hamster blood cells and is only present in trace amounts (1.2%).

In contrast to the large difference in PS species composition between whole worms and the tegument, there were no significant differences observed in PE species of the tegument compared to the whole worm (Table S1).

PC species analysis

The species composition of PC in schistosomes was clearly different from that of hamster blood cells (Table S2). For example, the number one in PC of the worms with 14% was 16:0/20:1, while that species represented less than 1% of the total PC in hamster blood cells. The most abundant PC species in hamster blood cells was 16:0/18:2 (20%), while this species ranked 5th and represented only 5% of the total PC species in the worms.

Striking, however, are also the differences in PC species composition between worms and their tegumental membranes. The tegument is twofold enriched in 16:0/16:0 diacyl PC, the most abundant PC species in these membranes (Table S2). The most remarkable difference between tegument and worms is the gross enrichment in 16:0/18:1 PC, where the 18:1 fatty acid is very unusual as we showed

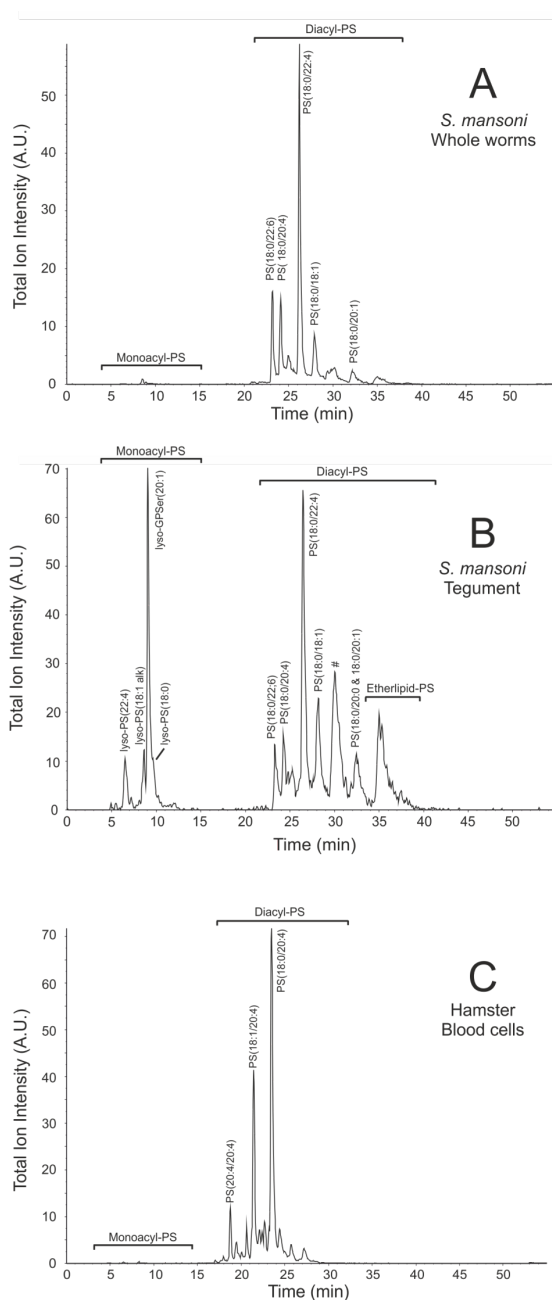


Figure 6.1: Analysis of the phosphatidylserine (PS) species composition by HPLC-mass spectrometry. Molecular species were determined in lipid extracts of homogenates of whole *S. mansoni* worms (panel A), of isolated tegument membranes from adult *S. mansoni* worms (panel B) and of hamster blood cells (panel C). Phosphatidylserine (PS) species were detected and quantified by multiple reaction monitoring using $[M-H]^-$ for MS1 and $[M-H-87]^-$ for MS2. Shown is the total ion intensity in arbitrary units during elution. Peak labels correspond to those in Table 6.1

Table 6.1: Molecular species of diacylphosphatidylserine (PS). Molecular species were determined in homogenates of whole adult *S. mansoni* worms, in isolated tegument membranes from adult *S. mansoni* worms and in hamster blood cells. PS species were quantified by MRM with [M-H]⁻ for MS1 and [M-H-87]⁻ for MS2. Percentages shown are the average percentages of the total PS species ± standard deviation, of species comprising more than 1% of the total PS. The three most abundant species in each membrane preparation are printed bold and species that comprise more than 2% of the total PS and are enriched at least twofold in tegument compared to lipids in whole worms are underlined in red. Species marked by an * comprise less than 1% of the total PS in that membrane preparation. Rt, retention time in minutes; #, not identified; \$, tentative identification based on m/z, retention time and head group fragmentation as full product spectra could not be obtained.

m/z	Rt	Acyl chains	Component	Whole worm (n=5) % ± S.D.	Tegument (n=3) % ± S.D.	Hamster blood cells (n=5) % ± S.D.
760	24.7	16:0/18:1	diacyl PS	*	*	1.0 ± 0.1
772	29.7	16:0/20:1	plasmalogen PS	1.9 ± 0.2	<u>5.2 ± 1.4</u>	*
782	21.7	16:0/20:4	diacyl PS	*	*	1.8 ± 0.2
784	22.7	18:1/18:2	diacyl PS	*	*	1.3 ± 0.1
786	25.1	18:0/18:2	diacyl PS	2.6 ± 0.2	1.5 ± 0.1	2.5 ± 0.1
788	28.5	18:0/18:1	diacyl PS	5.3 ± 0.5	3.2 ± 0.5	2.4 ± 0.3
796	22.8	17:0/20:4	diacyl PS	*	*	1.9 ± 0.1
798	31.4	#		1.6 ± 0.2	<u>3.8 ± 1.2</u>	*
800	35.8	18:0/20:1	plasmalogen PS	3.6 ± 0.6	<u>10.1 ± 2.9</u>	*
802	27.6	#		*	<u>2.0 ± 0.7</u>	*
804	29.6	38:0 \$	alkyl,acyl PS	*	<u>7.1 ± 0.7</u>	*
806	19.9	18:2/20:4	diacyl PS	*	*	2.4 ± 0.2
808	22.0	18:1/20:4	diacyl PS	*	*	14.8 ± 0.6
810	24.1	18:0/20:4	diacyl PS	5.4 ± 0.3	2.3 ± 0.3	36.0 ± 2.7
812	25.6	18:0/20:3	diacyl PS	2.0 ± 0.1	*	4.8 ± 0.3
814	28.1	18:0/20:2	diacyl PS	1.9 ± 0.1	1.3 ± 0.2	*
816	32.1	18:0/20:1	diacyl PS	3.5 ± 0.4	5.4 ± 0.3	*
818	32.1	18:0/20:0	diacyl PS	*	1.8 ± 0.1	*
822	29.0	#		1.3 ± 0.2	1.4 ± 0.2	*
824	25.3	19:0/20:4	diacyl PS	*	*	1.1 ± 0.1
830	19.2	20:4/20:4	diacyl PS	*	*	4.4 ± 0.5
830	29.8	#		*	<u>4.1 ± 0.5</u>	*
832	22.0	40 : 7	diacyl PS	*	*	3.0 ± 0.1
832	35.0	40 : 0	alkyl,acyl PS	*	<u>9.7 ± 1.4</u>	*
834	22.0	18:1/20:5	diacyl PS	*	*	1.9 ± 0.1
834	23.2	18:0/22:6	diacyl PS	7.6 ± 1.3	4.2 ± 0.2	3.5 ± 0.3
836	24.1	18:1/22:4	diacyl PS	*	*	1.9 ± 0.1
836	25.2	18:0/22:5	diacyl PS	5.2 ± 0.5	3.0 ± 0.9	1.2 ± 0.1
838	26.1	18:0/22:4	diacyl PS	34.3 ± 1.8	16.5 ± 2.2	2.8 ± 0.2
840	28.9	#		5.3 ± 0.2	2.6 ± 0.4	*
854	27.9	#		*	1.6 ± 0.5	*
864	27.0	#		1.7 ± 0.2	*	*
866	29.4	20:0/22:4	diacyl PS	1.1 ± 0.1	*	*
866	30.0	18:0/24:4	diacyl PS	1.4 ± 0.1	*	*

earlier that it contains the double bond at C5, instead of the common C9 position [22]. In tegumental membranes this 16:0/18:1 Δ^5 PC represented 12% of the total PC, while worm PC contained only 2%. This is in agreement with our earlier study where we showed that this rare PC species is not detectable in blood of the host and is, apart from dipalmitoyl PC, the most abundant PC species in the tegument of schistosomes [22].

PI species analysis

Analysis of the species composition of PI revealed that also large differences existed between the PI species composition of blood cells and that of the schistosome membranes (Table S3). Blood cells contain predominantly PI 18:0/20:4 and PI 18:0/18:2, representing together half of the PI species in hamster blood cells. On the other hand, membranes of whole worms as well as tegumental membranes contained in addition to PI 18:0/20:4, a large amount of PI 18:0/18:1 (21 and 27% in worms and tegument respectively), which represents in hamster blood cells only 2% of the total PI species (Table S3).

When compared to the differences in species compositions of PC and PS, the differences we observed in PI species composition are remarkably small between whole worms and tegumental membranes. PI species are well known to generate potent signaling molecules, but the absence of specific enrichment of certain PI species in the tegument, suggests that specific PI species are not involved in tegument-specific functions.

Analysis of lysophospholipids

Next to analysis of the diacyl-phospholipid species, we also investigated the presence of monoacyl-phospholipid (lyso-PL) species of the four above mentioned phospholipid classes, as it had been reported that lyso-PC as well as lyso-PS species are important in host-parasite interactions in schistosomiasis [69, 106, 187]. Lysophospholipids and diacyl-phospholipids do not ionize with the same efficiency, and therefore, these phospholipids are not detected with the same efficiency by mass spectrometry. For this reason, the molar ratio between lysophospholipids and diacyl-phospholipids cannot be estimated by mass spectrometry unless careful calibration is performed with a large array of standards, which are not commercially available. However, in order to determine whether one of the three investigated membrane fractions contains a relatively large proportion

Table 6.2: Ratio of lysophospholipids over diacylphospholipids. Molecular species were determined and quantified as described in the materials and methods in homogenates of adult *S. mansoni* worms, in isolated tegument membranes from adult *S. mansoni* worms and in hamster blood cells. Ratios shown are the average ratios of all detected lysophospholipid species over that of all diacylphospholipids species \pm standard deviation of each phospholipid class in arbitrary units where the ratio in whole worms was set at 1.0.

	Whole worms ratio \pm S.D. (n=5)	Tegument ratio \pm S.D. (n=4)	Hamster blood cells ratio \pm S.D. (n=4)
lyso-PS / diacyl-PS	1.0 \pm 0.2	15.8 \pm 4.0	2.3 \pm 2.9
lyso-PE / diacyl-PE	1.0 \pm 0.3	7.2 \pm 7.1	1.3 \pm 0.4
lyso-PC / diacyl-PC	1.0 \pm 0.4	1.6 \pm 0.9	1.4 \pm 0.5
lyso-PI / diacyl-PI	1.0 \pm 0.1	3.4 \pm 0.2	0.2 \pm 0.1

of lysophospholipid species, the ratio of the detected signals of lysophospholipid species over that of diacyl-phospholipid species can be compared between these fractions. Table 6.2 shows the ratio of detected signals derived from lysophospholipid species over that of diacyl-phospholipids for each phospholipid class in the three analyzed membrane preparations. These results are in line with earlier observations that the schistosomula of *S. mansoni* can produce lyso-PC [71], and we now show that in adult schistosomes the tegumental membranes, when compared to whole worms, are enriched in lyso-phospholipid species, especially lyso-PS and lyso-PE.

Analysis of the lysophospholipid species of the distinct phospholipid classes demonstrated that in blood cells the total acyl-chain length and number of desaturations of the minor amounts of lyso-PC, lyso-PE and lyso-PS were very similar to each other, because over 75% of the lysophospholipid species in these classes comprised acyl chains with 16 or 18 carbon atoms and none or a single desaturation (not shown). The lyso-PI species composition in blood cells also contained a large amount of lyso-PI 20:4 in addition to the lyso-PI species with acyl chains of 16 and 18 carbon atoms (not shown). On the other hand, membranes of adult *S. mansoni* worms had a lyso-phospholipid species composition with species containing unsaturated acyl chains of 16 or 18 carbon atoms and substantial amounts (over 20%) of lyso-PS 20:1 and lyso-PE 20:1 (Table 6.3). Interestingly, these two lysophospholipid species were drastically enriched in the tegumental membranes when compared to whole worms, and these species were by far the most abundant lyso-PS and lyso-PE species present in the tegumental membranes (Table 6.3). Eicosenoic acid (20:1) is synthesized by the parasite by

chain elongation of oleic acid (18:1) and it is an abundantly present fatty acid in schistosomes while virtually absent in the host [18, 20, 68]. The enrichment in the tegument of eicosenoic acid containing lyso-PS and lyso-PE is not observed in lyso-PC and lyso-PI, and the species composition of these lysophospholipid classes is dominated by species containing unsaturated acyl chains of 16 or 18 carbon atoms in lyso-PC and lyso-PI, respectively (Table 6.3). Therefore, no major differences exist in the lysophospholipid species composition of lyso-PC and lyso-PI between tegumental membranes and those whole worms, whereas the tegumental membranes are drastically enriched in lyso-PE and lyso-PS species containing eicosenoic acid (20:1).

Analysis of possible excretion of lysophospholipids by schistosomes

Because of the increased watersolubility of lysophospholipids compared to diacyl phospholipids, the specific enrichment of lysophospholipids in the tegumental membranes that form the outer-surface of the parasite, and because of the suggested function of lysophospholipids in host-parasite interactions [106, 146, 187], we investigated whether these lysophospholipids are excreted into their environment. Lysophospholipid secretion by schistosomes was examined *in vitro* by total phospholipid analysis of medium incubated with or without adult schistosomes. None of the schistosome-specific lysophospholipid or phospholipid species appeared to be present in the medium incubated with schistosomes, and no significant differences in species composition of PC, PE, PS and PI were detected between medium incubated with schistosomes and control medium (not shown).

The possible excretion of tegument specific lysophospholipids was also investigated *in vivo* by analysis of total phospholipids present in blood plasma of infected and non-infected hamsters. Again none of the schistosome-specific or tegument-specific lysophospholipids or diacylphospholipid species was increased in plasma derived from schistosome infected hamsters when compared to non-infected hamsters. These results demonstrate that the tegument-specific lyso-phospholipids are not significantly secreted into aqueous solutions with albumin and do not circulate in blood of infected hosts. However, a substantial decrease in total PI content was detected in plasma from infected hamsters compared to non-infected hamsters (Table 6.4). All these 5 PI species are common and abundant diacyl PI species in host blood cells (Table S3).

Table 6.3: Molecular species of lyso-phospholipids. Molecular species were determined in lipid extracts of homogenates of whole *S. mansoni* worms and isolated tegument membranes from *S. mansoni* worms. Lyso-PS species were quantified by MRM with [M-H]⁻ for MS1 and [M-H-87]⁻ for MS2. Lyso-PE species are quantified by MRM with [M+H]⁺ for MS1 and [M+H-141]⁺ for MS2. Lyso-PC species are quantified by MRM with [M+H]⁺ for MS1 and m/z 184 for MS2. Lyso-PI species are quantified by MRM with [M-H]⁻ for MS1 and m/z 241 for MS2. Percentages shown are the average percentages of the total detection of lysophospholipid species ± standard deviation, of species comprising more than 1% of the total lysophospholipids. Species marked by an * comprise less than 1.0% of the total lysophospholipids in that membrane preparation. Species containing an ether-linkage are indicated by the addition alk and the most abundant lysophospholipid species in each fraction is printed bold. Species that are enriched at least twofold in tegument compared to whole worms are underlined in red.

Fatty acyl	m/z	Lyso PS (% ± S.D.)		m/z	Lyso PE (% ± S.D.)		m/z	Lyso PC (% ± S.D.)		m/z	Lyso PI (% ± S.D.)	
		Worms (n=5)	Tegument (n=5)		Worms (n=5)	Tegument (n=5)		Worms (n=5)	Tegument (n=5)		Worms (n=5)	Tegument (n=5)
16:0	496	1.5 ± 0.3	2.5 ± 1.1	454	7.1 ± 4.7	7.7 ± 3.3	496	38.4 ± 4.0	47.8 ± 3.1	571	1.8 ± 0.9	<u>5.6 ± 1.1</u>
18:1 alk	508	7.6 ± 2.3	4.1 ± 3.8	466	*	*	508	*	*	583	*	*
18:0 alk	510	2.1 ± 0.6	*	468	*	*	510	*	*	585	5.3 ± 0.6	2.6 ± 1.1
18:2	520	*	*	478	*	*	520	2.4 ± 1.0	2.6 ± 1.8	595	*	<u>4.5 ± 0.8</u>
18:1	522	1.3 ± 0.7	1.5 ± 0.4	480	8.3 ± 4.2	11.5 ± 2.2	522	9.1 ± 1.7	11.4 ± 3.1	597	3.2 ± 2.0	<u>11.7 ± 3.6</u>
18:0	524	46.2 ± 4.4	9.3 ± 4.2	482	63.1 ± 8.5	19.8 ± 9.5	524	27.5 ± 2.2	23.9 ± 5.9	599	80.2 ± 4.3	62.8 ± 4.8
20:4	544	*	*	502	*	*	544	2.6 ± 0.3	*	619	1.3 ± 0.4	<u>3.1 ± 1.0</u>
20:2	548	*	*	506	*	*	548	3.6 ± 1.2	3.1 ± 1.3	623	*	1.5 ± 0.9
20:1	550	24.0 ± 4.5	<u>62.6 ± 7.2</u>	508	21.6 ± 10.4	<u>57.1 ± 8.1</u>	550	9.0 ± 1.1	8.0 ± 3.6	625	*	<u>2.0 ± 0.9</u>
20:0	552	2.1 ± 0.4	2.6 ± 0.4	510	*	<u>1.4 ± 2.2</u>	552	2.3 ± 0.3	*	627	*	*
22:4	572	8.1 ± 1.4	10.1 ± 0.7	530	*	*	572	1.3 ± 0.7	*	647	*	*
22:1	578	*	1.3 ± 0.7	536	*	*	578	*	*	653	*	*
24:1	606	1.3 ± 0.2	*	564	*	*	606	*	*	681	5.2 ± 1.4	4.4 ± 1.2

Table 6.4: Phospholipid species with a significantly different abundance in blood plasma of *Schistosoma mansoni* infected versus non-infected control hamsters. Molecular species of PC, PE, PS and PI were determined in blood plasma of hamsters infected with *S. mansoni* or non-infected control hamsters. Species were quantified by MRM as described in material and methods and the legends of Table 6.1 and Tables S1-S3. The five phospholipid species are shown that differ most significantly, at least twofold in abundance in blood plasma of infected hamsters compared to non-infected control hamsters and represent at least 0.4% of the total phospholipid signal.

m/z	PL class	Acyl chains	Ratio infected/control	P-value
833	PI	34:2	0.35	$1.17 \cdot 10^{-7}$
861	PI	36:2	0.37	$5.46 \cdot 10^{-6}$
857	PI	36:4	0.38	$1.21 \cdot 10^{-6}$
887	PI	38:3	0.40	$4.96 \cdot 10^{-5}$
885	PI	38:4	0.42	$2.61 \cdot 10^{-6}$

6.4 Discussion

The tegumental surface membranes of adult schistosomes, a unique biological structure which forms the site of interaction with the host [113], comprises many schistosome-specific and tegument-specific lipids. In addition to the already reported tegument-specific enrichment of several PC and ether-linked PE species compared to whole worms [18, 22], our results showed that also multiple PS species are specifically enriched in the tegument. On the other hand, no tegument-specific PI species could be detected. The species composition of the most abundant phospholipid classes (PC and PS) in tegumental membranes differs drastically from that of whole worms. However, this phenomenon is not true for all phospholipid classes, as the species compositions of PI and PE in tegumental membranes did not differ from those of whole worms. Furthermore, the enrichment in the tegument of eicosenoic acid (20:1) containing lysophospholipids was only observed for PS and PE and not for PC and PI. These results, therefore, suggest that the tegument comprises mechanisms that facilitate the enrichment of certain phospholipids in the membranes of the tegument. The function of most of the identified tegument-specific phospholipids and lysophospholipids is not yet known, but the lyso-PS and lyso-PC species of schistosomes have been shown to activate TLR-2 on host immune cells, resulting in a down-regulation of the host immune response and eosinophil activation and recruitment, respectively [106, 146, 187].

Interestingly, the various phospholipid species enriched in the tegument of the distinct phospholipid classes do not contain a similar set of acyl chains, as different species for PC and PS are enriched in the tegument (Table 6.1 and

supplementary data). Hence, the tegumental membranes are enriched in specific phospholipid species that differ between the distinct phospholipid classes.

Earlier observations on a different life cycle stage, schistosomula, clearly demonstrated that this stage exports home made lyso-phospholipids [69]. It is unknown yet whether adult schistosomes excrete the schistosome-specific and/or tegument-specific phospholipids. Detection of the possible excretion of these lipids is hampered by the biophysical properties of the lipids in question, as they contain relatively hydrophobic long-chain fatty acids and lysophospholipids that can be expected to partition quickly into nearby phospholipid membranes. *In vivo*, the tegument-specific lysophospholipids might spread quickly to nearby membrane structures, which would then allow receptor activation of host immune cells as reported in *in vitro* studies [106, 146, 187]. Knowing this, we nevertheless searched specifically for excretions of those schistosome-specific lipids by adult schistosomes. We could, however, not detect any significant secretion of these lipids by schistosomes, not during our *in vitro* incubations, nor in the blood of infected hamsters. Therefore, the *in vivo* mechanism by which these lysophospholipids might fulfil the *in vitro* observed effects on host immune cells remains to be resolved and probably requires sophisticated co-culture studies that are beyond the scope of this study.

However, analysis of the phospholipid composition in blood plasma of infected and non-infected hamsters demonstrated another interesting difference, as a substantial decrease in PI content was observed in blood plasma of schistosome infected hamsters. As phospholipids are not bound to albumin, because this protein binds predominantly fatty acids, these phospholipids probably constitute small vesicular structures, such as exosomes. The substantial decrease in PI is peculiar as this phospholipid is well known for its precursor function in signal transduction. Therefore, these results might indicate that the schistosome infection alters exosome formation by endothelial cells or by circulating immune cells, which is under current investigation.

Supplementary data

Figure S1 (following page): Discriminant analysis of the phosphatidylserine (PS) species composition.

Molecular species were determined in lipid extracts of homogenates of whole *S. mansoni* worms, of isolated tegumental membranes from *S. mansoni* worms and of hamster blood cells. Panel A shows the score plot of the discriminant analysis, in which the differences in PS species composition between hamster blood cells and schistosome membranes (from both whole worms and tegumental membranes) become visible on the X-axis (discriminant 1, D1), whereas the differences in PS species composition in tegumental membranes compared to those of whole worms are visible on the Y-axis (D2, discriminant 2). Panel B shows the loading plot, in which the detected PS species are plotted at the location corresponding with their value in discriminant 1 and 2. The PS species that differ most between the three membrane preparations are labeled with their m/z ratio @ retention time, as listed in Table 6.1.

This discriminant analysis revealed that the differences among the independent replicates of the three investigated membrane preparations (whole worms, tegument and host blood cells), were much smaller than the differences between the three distinct membrane preparations (Fig. S1A). Discriminant 1 (represented by the X-axis in panel A) indicates the differences in PS species between blood cells and those of schistosomes, whereas discriminant 2 (represented by the Y-axis in panel A) indicates the differences in PS between tegumental membranes and those of whole adult worms. In the corresponding loading plot (panel B) all PS species are represented by a dot and those PS species that do not vary in abundance between the distinct membrane preparations are plotted close to the origin of the X-axis and Y-axis, as they hardly contribute to the distinction between the three membrane preparations. On the other hand, the PS species that vary in abundance between blood cells and those of schistosomes are plotted far from the origin of the X-axis. For instance, PS 18:0/20:4 and PS 18:1/20:4 with a m/z ratio of 810 and 808, respectively, are the two most abundant PS species in blood cells and present in very low amounts in both schistosome samples (Table 6.1). Hence, these species are major discriminating PS species between blood cells and schistosome samples, and therefore, these PS species are located in the loading plot of the discriminant analysis at a position with a high loading on discriminant 1 (Fig. S1B, on the X-axis, far right in bottom corner). Similarly, PS 18:0/22:4 (m/z = 838), which is abundantly present in lipid samples of whole adult schistosomes, less present in tegumental membranes and far less present in blood cells (Table 6.1), is plotted at with a high loading on both discriminant 1, representing the differences between blood cells and schistosome lipids (Fig. S1B, X-axis, far left), as well as with a high loading on discriminant 2, representing the differences between the PS species in tegumental membranes and those of whole adult worms (Fig. S1B, Y-axis, far to the bottom).

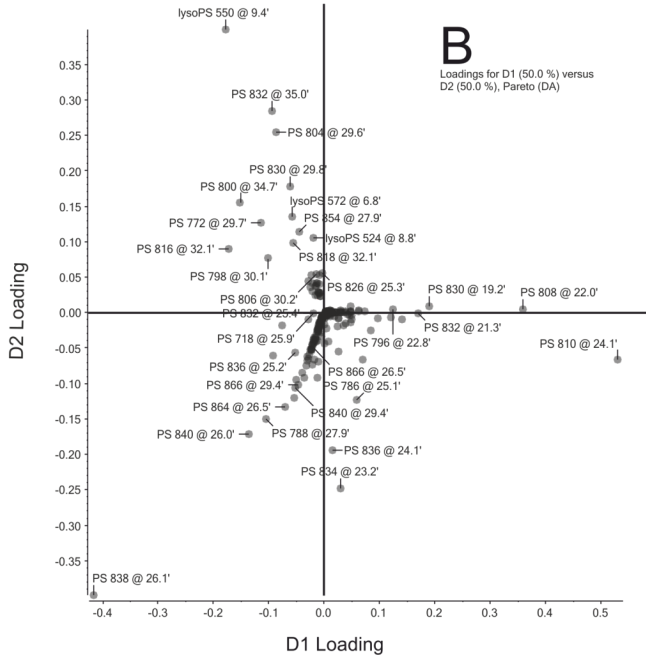
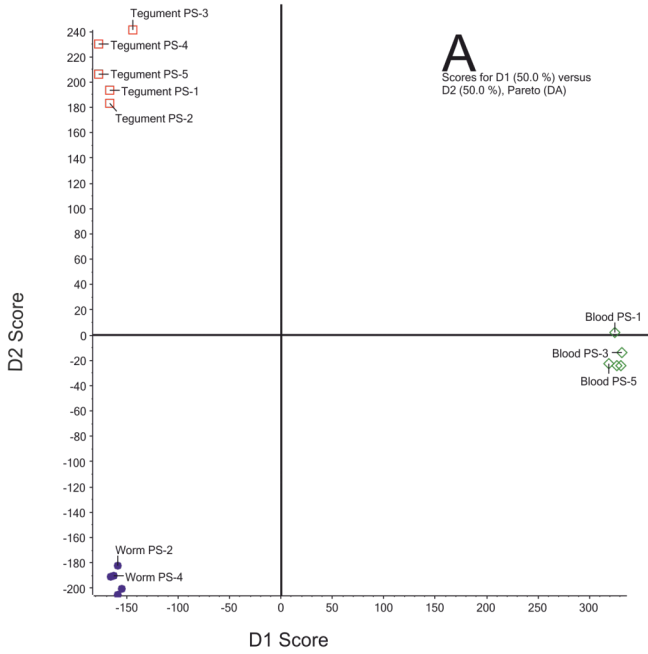


Table S1: Molecular species of phosphatidylethanolamine (PE). Molecular species were determined in homogenates of adult *S. mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood cells. Species are quantified by MRM with $[M+H]^+$ for MS1 and $[M+H-141]^+$ for MS2. Percentages shown are the average percentages of the total PE species \pm standard deviation. Species are shown if these were among the 10 most abundant species in one of the three examined membrane preparations. The three most abundant species in each fraction are printed in bold and species marked by an * comprise less than 1% of the total PE of that membrane preparation.

m/z	Acyls	Whole worm (n=5) % \pm S.D.	Tegument (n=3) % \pm S.D.	Hamster blood cells (n=5) % \pm S.D.
718	16:0/18:1	2.8 \pm 0.3	5.3 \pm 0.5	7.5 \pm 0.5
740	16:0/20:4	1.2 \pm 0.2	1.5 \pm 0.2	6.4 \pm 0.2
742	18:1/18:2	2.1 \pm 0.3	2.5 \pm 0.2	7.4 \pm 0.4
744	18:1/18:1	7.1 \pm 0.6	8.4 \pm 0.3	6.6 \pm 0.2
746	18:0/18:1	8.2 \pm 0.3	9.5 \pm 1.0	3.9 \pm 0.2
764	18:2/20:4	1.2 \pm 0.1	1.1 \pm 0.2	6.4 \pm 0.1
766	18:1/20:4	2.8 \pm 0.1	2.9 \pm 0.2	10.1 \pm 0.1
768	16:0/22:4	4.6 \pm 0.2	6.1 \pm 0.8	3.3 \pm 0.1
768	18:0/20:4	4.1 \pm 0.2	2.3 \pm 0.3	4.8 \pm 0.1
770	18:2/20:1	5.5 \pm 0.1	6.4 \pm 0.4	1.8 \pm 0.1
772	18:1/20:1	3.9 \pm 0.1	5.5 \pm 0.4	*
788	20:4/20:4?	*	*	5.1 \pm 0.3
790	18:1/22:6?	2.7 \pm 0.1	2.0 \pm 0.0	6.6 \pm 0.2
792	18:1/22:4	4.8 \pm 0.4	3.1 \pm 0.5	4.0 \pm 0.1
794	18:0/22:5	6.3 \pm 0.5	5.5 \pm 0.8	3.8 \pm 0.3
796	18:0/22:4	17.9 \pm 1.0	15.0 \pm 2.1	1.2 \pm 0.1
798	18:0/20:3	4.3 \pm 0.4	4.7 \pm 0.3	*
818	20:1/22:6	4.5 \pm 0.5	3.2 \pm 0.2	*

Table S2: Molecular species of phosphatidylcholine (PC). Molecular species were determined in homogenates of adult *S. mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood cells. Species are quantified by MRM with $[M+H]^+$ for MS1 and m/z 184 for MS2. Percentages shown are the average percentages of the total PC species \pm standard deviation. Species are shown if these were among the 10 most abundant species in one of the three examined membrane preparations. The three most abundant species in each fraction are printed in bold and species that are enriched at least twofold in tegument compared to lipids in whole worms are underlined in red. Species marked by an * comprise less than 1.0% of the total PC in that sample. Of unidentified species the attached acyl-chains are given in total number of carbon atoms and unsaturations. Species containing an ether-linkage are indicated by the addition alk.

m/z	Acyls	Whole worm (n=5) % \pm S.D.	Tegument (n=3) % \pm S.D.	Hamster blood cells (n=5) % \pm S.D.
720	16:0/16:0 alk	1.5 \pm 0.1	<u>5.8 \pm 1.3</u>	*
734	16:0/16:0	11.5 \pm 0.7	<u>23.1 \pm 2.2</u>	6.3 \pm 0.2
746	34:1 alk	1.8 \pm 0.1	2.2 \pm 0.3	*
758	16:0/18:2	4.8 \pm 0.4	5.1 \pm 0.2	19.8 \pm 1.1
760	16:0/18:1 Δ^9	11.7 \pm 0.6	8.2 \pm 0.2	14.4 \pm 0.69
760	16:0/18:1 Δ^5	2.2 \pm 0.1	<u>12.4 \pm 0.3</u>	*
762	16:0/18:0	4.6 \pm 0.2	3.9 \pm 0.1	2.5 \pm 0.1
782	16:0/20:4	3.5 \pm 0.4	2.2 \pm 0.7	6.6 \pm 0.3
784	18:1/18:2	2.4 \pm 0.1	2.3 \pm 0.1	3.8 \pm 0.2
786	18:0/18:2	10.1 \pm 0.3	7.0 \pm 0.5	11.9 \pm 0.6
788	16:0/20:1	13.8 \pm 0.4	5.7 \pm 0.5	*
788	18:0/18:1	*	*	3.7 \pm 0.3
806	16:0/22:6	2.0 \pm 0.2	1.4 \pm 0.3	6.9 \pm 0.6
808	18:1/20:4	1.5 \pm 0.1	*	3.0 \pm 0.2
810	18:0/20:4	2.4 \pm 0.2	1.3 \pm 0.2	4.1 \pm 0.3

Table S3: Molecular species of diacylphosphatidylinositol (PI). Molecular species were determined in homogenates of adult *S. mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood cells. Species are quantified by MRM with $[M-H]^-$ for MS1 and m/z 241 for MS2. Percentages shown are the average percentages of the total PI species \pm standard deviation. Species are shown if these were among the 10 most abundant species in one of the three examined membrane preparations. The three most abundant species in each sample are shown in bold and species that are enriched at least twofold in tegument compared to lipids in whole worms are underlined in red. Species marked by an * comprise less than 1.0% of the total PI. Of unidentified species the attached acyl-chains are given in total number of carbon atoms and unsaturations.

m/z	Acyls	Whole worm (n=5) % \pm S.D.	Tegument (n=3) % \pm S.D.	Hamster blood cells (n=5) % \pm S.D.
833	16:0/18:2	4.0 \pm 1.6	2.2 \pm 0.2	7.1 \pm 0.7
835	16:0/18:1	3.1 \pm 0.6	3.7 \pm 0.6	2.4 \pm 0.4
837	16:0/18:0	2.3 \pm 0.6	<u>6.3 \pm 0.9</u>	*
857	16:0/20:4	1.1 \pm 0.1	*	6.6 \pm 0.6
859	18:1/18:2	1.3 \pm 0.0	1.2 \pm 0.2	3.4 \pm 0.2
861	18:0/18:2	15.7 \pm 1.3	13.2 \pm 1.3	13.0 \pm 0.7
863	18:0/18:1	21.0 \pm 2.5	27.3 \pm 1.9	2.3 \pm 0.1
865	18:0/18:0	2.3 \pm 0.3	2.7 \pm 0.1	*
883	18:1/20:4	*	*	5.2 \pm 0.1
885	18:0/20:4	25.0 \pm 1.9	9.9 \pm 0.8	36.8 \pm 2.1
887	18:0/20:3	3.4 \pm 0.4	3.9 \pm 0.6	5.7 \pm 0.3
889	38:2	2.2 \pm 0.2	4.3 \pm 0.6	1.2 \pm 0.2
891	18:0/20:1	1.4 \pm 0.1	<u>3.3 \pm 0.5</u>	*
911	40:5	*	*	2.8 \pm 0.3
945	42:2	*	<u>2.3 \pm 0.2</u>	*

Chapter 7

Summarizing discussion

7.1 Development of parasitism

Parasitism is a relationship between two organisms in which one of them (the parasite) lives in or on the other (the host) and lives at the expense of that host. Life as a parasite offers advantages that surpass its inconveniences. The main advantage is the excess of nutrients. In addition, parasitism offers the possibility to lose genes encoding proteins that are essential under free-living conditions, but of which the function is taken over by the host. A parasite also benefits from the homeostasis of the host, giving the parasite a very constant environment to live in. However, living in or on a host organism also means living in constant threat of the host immune system. Another disadvantage is the more challenging way of reproduction, which is reflected in the numerous complex life cycles of parasites.

Parasitism has evolved independently many times during the history of life. Almost all free-living organisms are host to various parasitic taxa [139]. There are parasites in nearly all ecosystems and there is substantial evidence that parasites shape host population dynamics, alter interspecific competition, influence energy flow and appear to be important drivers of biodiversity [86].

A parasite's way of living inevitably causes damage to its host, if not physical, than by taking up nutrients that would otherwise be for the host itself. Parasites generally die when their host does. Hence, parasites have evolved in such a way that the damage they cause will not kill their host, at least not before the parasites have reproduced. The harm caused by schistosomes is usually mild. They can cause anemia because they feed on blood, but also as a result of blood loss through the wounds made by the eggs when they pass the endothelium and gut wall. Schistosome infections can also lead to malnutrition. The eggs that do not exit the body, elicit an immune response that initially is not very harmful to the host but can be detrimental when it is at a critical location, e.g. the central nervous system (CNS), or after long lasting accumulation causing portal hypertension and which may lead to liver failure. Also, prolonged inflammation may contribute to the development of malignancies.

Parasites adapted to nearly every kind of tissue of the (human) body exist. Each parasite has evolved for optimal fitness within its niche. Schistosomes are blood-dwelling parasites. The worms have to overcome many challenges in order to get into the circulation, maintain themselves, reproduce and to excrete the eggs for survival of their species. They have developed a large armoury of interventions

with their host to protect themselves and sometimes even to turn potential harmful host reactions in highly useful assistance for example in excretion of the eggs.

7.2 Parasitic helminths

Infection and migration

Penetration of the skin is a fairly common mode of entrance for parasitic worms. This can be achieved either by a vector injecting worm larvae or by skin penetration of the larval parasites themselves. Filaria worms are injected through the skin by flies or mosquitoes. Hookworm and *Strongyloides* larvae penetrate the skin by themselves, as do schistosome cercariae.

Tissue migration is a common feature in the life cycles of parasitic worms. Similar to schistosomula, both hookworm and *Strongyloides* larvae migrate to the lungs. However, while schistosomula further migrate via the liver to the portal vein to mate and mature, hookworm and *Strongyloides* larvae enter the alveoli, are coughed up and subsequently swallowed to reach their final location, which is the intestinal lumen where they mature and reproduce. And even *Ascaris* larvae, which enter the human host when eggs are ingested, need to penetrate the mucosa of the gut and migrate through the body in order to fully mature before homing in the intestinal lumen – where they have been before as eggs. Thus, although tissue invasion and migration appears as a redundantly complicated step in the life cycle of parasitic worms, it is so widespread that it seems to provide essential developmental needs.

Tissue migration is referred to as larva migrans when it causes disease symptoms. As can be deduced from the life cycles of the helminths that cause larva migrans, symptoms are temporally and disappear once the adult worms have reached their niche. Only upon recurrent reinfection or in the case of *Strongyloides*, where auto-infection can occur, larva migrans can last for longer periods. Tissue migration of schistosomes doesn't usually cause symptoms, even though it elicits a remarkable immune response.

Although very common, not all parasitic worms migrate through tissues. *Enterobius* and *Trichuris* are both ingested as eggs and hatch and develop to adult worms within the gut. And also for the flatworms *Opisthorchis viverrini*, *Clonorchis sinensis* and *Dicrocoelium dendriticum* the only organ system they encounter from their host, is the intestinal tract and the bile duct.

On the other hand, not all ingested parasitic worms home in the intestinal tract. Some are ingested as eggs or as larvae and migrate to other tissues to mature and reproduce. *Dracunculus* are ingested with their intermediate hosts, copepods, in drinking water. Larvae, released from the copepods which die in the stomach, penetrate the host stomach and intestinal wall to mature and copulate in the abdominal cavity and retroperitoneal space. The female worms then migrate to the subcutaneous tissues towards the skin surface where larvae are released through a skin blister. Also *Trichinella* and *Echinococcus* are parasitic worms that infect humans through ingestion. They then migrate throughout the body where cysts are formed, which only infect the next host through carnivorism, when ingested with the current host as a meal for the new host.

Exit of progeny

In order to replicate, the progeny of parasitic worms needs a way to exit the host and infect another host. The majority of human parasitic worms spreads through eggs or larval stages that leave their host. For intestinal parasites, the route of exit is obvious. No complex mechanisms need to be adopted as eggs or larvae, which do not attach to the gut wall, will automatically be excreted following peristaltic bowel movements and defecation. *Schistosoma mansoni* also uses this mechanism, but first has to overcome some barriers to get their progeny into the gut. As oviposition occurs in the veins, eggs have to pass the endothelial wall and the gut wall. As for intestinal worms, the exit of eggs is with the feces. Miracidia, schistosome larvae, hatch from the eggs immediately when they come into fresh water. Time to infect the intermediate host, an aquatic snail, is then limited. Some parasitic worms, such as *Fasciola*, *Ascaris* and *Echinococcus* have very resistant eggs or cysts which can stay viable in the environment for a long time. Hatching occurs when favourable conditions are met, followed by infection of the next (intermediate) host.

Free-living stages of parasitic worms are usually non-feeding and thus are either encysted such as the metacercariae of *Fasciola* spp., or have a restricted time frame to infect the next host. The latter is the case for schistosomes, where both the miracidia infecting the snail and the cercariae infecting the mammalian host have a glycogen reserve for about 12–24 hours [89, 182, 202]. Hookworm and *Strongyloides* have free-living stages that feed on soil bacteria (rhabditiform larvae). When transformed into non-feeding, infective filariform larvae they encounter the

same time challenge of finding a suitable host as do schistosomes. *Strongyloides* eggs hatch within the uterus of the female worm. As transformation into infectious filariform larvae takes less than two days, this can occur within the host, before the larvae are excreted. The larvae can reinfect the host, which is referred to as auto-infection. In severely immunocompromised hosts, aberrant auto-infection may lead to hyperinfection syndrome. *Strongyloides* also have a free-living cycle, where rhabditiform larvae mature into reproductive male and female worms.

As mentioned above, intestinal parasites have an easy exit. Tissue-dwelling parasitic worms on the other hand need to be more inventive to exit and spread. Carnivorism is a well proven concept for life cycle continuation, used for example by some tapeworms (*Taenia solium*, *Echinococcus*) and *Trichinella*. Filaria larvae, microfilaria, circulate within the bloodstream and are aspirated and further spread to their new host by the fly or mosquito vector upon feeding. Microfilariae from tissue-dwelling filariae generally hatch in the uterus of female worms, while for blood-dwelling species microfilariae remain sheathed in the envelope of the egg and will exsheath within the intermediate host. *Dracunculus* worms do not lay eggs, but produce live larvae. These leave the host through a sore blister in the skin, usually the foot, which is immersed in water to ease the pain.

Toxocara is an intestinal parasitic worm in dogs and cats. Similar to many intestinal parasites, eggs leave with the feces. Worms can mature and reproduce in young dogs. However, in infected adult dogs, *Toxocara* development is hampered. But the infection can be transferred transplacental, which means that an infected pregnant dog can infect her unborn pups *in utero*. In addition, an infected bitch can infect her newborns through milk. *Toxocara* development is then resumed in young dogs.

Feeding habits

Most parasitic worms that infect humans feed on tissue, blood or other body fluids, even when residing in the intestinal tract. In that respect, schistosome worms have chosen a very pleasant location where food literally drifts by. All they need to do in order to get fed, is sit and open their mouth or absorb through their tegument. And that is exactly what they do. Female schistosome worms feed largely on circulating red blood cells. Males mainly absorb nutrients through their tegument. Tissue-dwelling worms generally feed on the tissue and body fluids they live in. Some of them eventually encyst and go into dormant stages and spread

through carnivorism. Hookworms and *Strongyloides*, both intestinal worms, hook themselves to the mucosa to feed on blood and epithelial cells. Where hookworms just hook themselves in the mucosa, *Trichuris* worms dig in half their body, while the other half of their body hangs freely in the gut lumen.

Some intestinal worms do not feed on human tissue but feed on the predigested intestinal liquid content. Among them are *Ascaris*, *Toxocara* and tapeworms. The latter have no mouth and absorb all the nutrients through their tegument, which is covered by microvilli to increase surface area.

Whichever locations parasitic worms chose, they generally have plenty of food surrounding them as they typically feed on the host. This allows them to use an inefficient energy metabolism in which substrates are not fully oxidized.

Host defence

The components of the immune system of the host are a continuous threat to the parasites living in the host. In that respect, the choice for the circulation as a habitat of schistosomes is remarkable, as this is the body compartment where all immune components are constantly present: immune cells, including T-cells, eosinophils and macrophages and other immune components such as complement, cytokines and chemokines. The presence of the immune effectors is much lower in the tissues and even lower in the gut. However, as parasitic worms are large compared to the immune cells, there is not much these immune cells can do to them even with help of other components of the immune system. Besides, many tissue-dwelling parasitic worms have developed effective immune evading strategies. Sometimes the immune activation results in encapsulation of the parasite (larvae), as is the case with larvae of species that infect through carnivorism such as the cyst stage of *Echinococcus* and *Taenia solium* and with schistosome eggs that are trapped in the body.

7.3 On schistosome eggs and worms

Eggs and worms of schistosomes have opposite interests, namely get out of the host versus stay in the host. Thus egg-host interactions and worm-host interactions have to be different.

Schistosome worms have evolved to produce a large progeny in order to maximize the probability of species survival. The adult worms do not look after their

offspring once the eggs are laid. Eggs have to get out of the host on their own, even though they are incapable of moving by themselves and the way out is a difficult one. Eggs have to penetrate tissues in order to get to the gut lumen (urine bladder in the case of *S. haematobium*) where they are subsequently released in the environment together with the feces (or urine).

Egg-host interactions

An egg is defined as an animal reproductive body consisting of an ovum/female gamete together with its nutrient material and a protective covering and, when fertilized, having the capacity to develop into a new individual capable of independent existence.

Schistosome eggs are build of a fertilized oocyte surrounded by vitelline cells containing nutrients and material for the eggshell [175]. By the time the egg leaves the mammalian host, the fertilized oocyte has developed into a fully matured miracidium covered by a tanned protein eggshell. Once the egg is released in the water, osmotic changes result in swelling of the internal material thereby increasing the internal pressure on the eggshell. This leads to hatching of the miracidium from the egg.

Formation of the eggshell

A major function of the eggshell is to restrict the permeability of the egg and maintain a certain environment within the egg for embryonic development and survival [196]. Eggshells of trematodes are made of proteins which are cross-linked by tyrosinase activity forming quinone bonds. This eggshell of tanned proteins has underlying layers and envelopes. The subshell cellular layer of schistosome eggs is named von Lichtenberg's envelope. A non-cellular layer, Reynolds' layer, is situated between von Lichtenberg's envelope and the eggshell [6, 123]. Schistosome eggshell formation starts in the ootype within the female, where ootype contraction leads to release of eggshell precursor proteins from the vitelline droplets. These form the eggshell upon quinone tanning. Specific eggshell precursor proteins have first been described in the late 80s and early 90s [13, 14, 33, 93, 94, 150, 169]. As the eggshell is a direct site of interaction between the egg and the host, its composition is of major importance in host-parasite interaction. Results of mass spectrometry, immunohistochemical and amino acid analysis of the protein composition of the eggshell are described in **chapter 2**.

The experiments revealed that the major component of the eggshell is the putative eggshell protein p14. This protein has characteristic short repeat sequences of which nearly half of the amino acid residues are glycines. High glycine content is a general feature of trematode eggshell proteins and has also been described for *Fasciola hepatica*, *Opisthorchis viverrini* and *Paragonimus westermani*. Mass spectrometry analysis did not reveal the presence of p14 in eggshell, probably due to its high tyrosine content transformed in quinone bonds and due to high lysine levels leading to very short peptides after trypsin digestion. However, the presence of p14 was shown by immunoblot. In addition, amino acid analysis showed a glycine content of 36% in total hydrolyzed eggshell. This high glycine content is readily explained by a high contribution of p14 to total eggshell.

A wide range of schistosome proteins was detected by mass spectrometry in purified eggshell samples. This indicates that a variety of non-eggshell specific proteins is also incorporated in the eggshell. The majority of these proteins are generally abundant cellular proteins such as the major egg antigen p40, HSP70 and many glycolytic enzymes. These proteins are suggested to originate from surrounding vitelline cells and happened to be around at the site and time of eggshell synthesis. Hence, these non-eggshell specific proteins were coincidentally cross-linked to the major eggshell proteins. The absence of the major egg secretion proteins IPSE/alpha-1 and omega-1 indicates that cross-linking is finished before the egg has fully matured and starts secreting. The absence of host plasma proteins in eggshell indicates that eggshell formation is finished even before oviposition.

Eggshell contribution to the journey of the *S. mansoni* egg

Most eggs of parasitic helminths are deposited in the gut. Their way out is straightforward and does not need exploited assistance of the host. In contrast, schistosome eggs exit the female body in the mesenteric capillary venules, close to the bowel. It takes at least a few days for a schistosome egg to exit the host, but it may take up to several weeks. The eggs need to get into the gut in order to get out of the host. However, blood flowing through the venules can easily carry the egg along, away from the bowel and heading towards the liver. To prevent this, eggs have to quickly adhere to the vessel wall. Previous experiments reported vast adhesion of platelets to eggshell [203]. In addition, the experiments in **chapter 4** showed that VWF also adheres to eggshell directly. Unfolded VWF can connect platelets to clotting factors and injured surfaces of the endothelium through many binding domains [105, 159] and plays an important role in platelet-vessel wall adhesion by

binding both platelets and the extracellular matrix of the vessel wall containing collagen and fibrin. Furthermore, VWF plays a role in platelet cross-linking and platelet plug expansion [105]. By bridging between the eggshell and the extracellular matrix, VWF may attach the eggshell to the endothelium. In addition, VWF can contribute to eggshell adhesion to endothelium indirectly as VWF in combination with platelets can induce platelet adhesion, platelet activation and secondary hemostasis, allowing the formation of a stable clot. This is in accordance with the findings of Ngaiza and Doenhoff (1990) [125] who previously demonstrated that platelets play a role in egg extravasation.

Binding of fibrinogen and fibronectin to eggshell was also demonstrated. Where fibrin, the cleaved product of fibrinogen, is the main component of a blood clot, fibronectin plays an essential role in cell adhesion and wound healing. Thus, both can play an important role in egg adhesion to the endothelium. Enolase was previously identified in eggshell. Its presence there seems accidental. Enolase has been described to bind host fibronectin in bacterial pathogens [57]. This could also be the case in *S. mansoni* eggshell, where enolase-binding fibronectin can further enhance endothelium binding of eggshell. **Chapter 4** reports that fibronectin was one of the main plasma proteins that bound to eggshell and the major one in the high molecular weight range. No tests were performed to determine whether enolase was the major fibronectin-binding eggshell protein.

Next to fibronectin, enolase can bind plasminogen [8, 43, 141, 206]. Plasminogen is the precursor of plasmin, a serine protease that cleaves fibrin and VWF. Small amounts of plasminogen may facilitate fibrinolysis of the clot surrounding the eggs after extravasation. However, in the mass spectrometry experiments plasminogen was not elevated in plasma proteins that bound to eggshell compared to full plasma. This does not exclude that plasminogen is bound to eggshell. In fact, small amounts of plasmin are probably needed to prevent the excessive expansion of the clot leading to occlusion of the vessel.

Platelet activation and blood coagulation around schistosome eggs can further be enhanced by shear stress and turbulence within the vessel [177]. Both can be caused by mechanical obstruction by worms and eggs. By their presence and as a result of their adherence to the vessel wall, schistosome worms also cause damage to the endothelium, which in itself is another strong activator of platelets and blood coagulation and of endothelial cells [168]. Eggs of both *S. mansoni* and *S. haematobium* can induce endothelial cell proliferation *in vitro* [55, 67]. *In vitro*, endothelial cells overgrow schistosome eggs within a few hours after oviposition

[64]. Thus, eggs can passively cross the endothelium by the non-specific response of endothelial cells. File (1995) [64] also showed that eggs deposited directly by adult worms elicited a more rapid and complete response than embryonated eggs isolated from the liver tissues.

In short, schistosome eggs can probably passively pass the endothelium after binding it. Binding of the eggshell is facilitated by the composition of the eggshell (**chapter 2 and 3**) which binds platelets and plasma proteins involved in coagulation (**chapter 4**).

The next step is to cross the tissue of the gut wall and get excreted with the feces. The mechanism of this process are largely unknown, but it is likely to depend on the host immune system (see below).

Eggshell interaction with the immune system

Most eggs of parasitic helminths are deposited in the gut and have little interaction with the immune system. In contrast, schistosome eggs are in constant interaction with the host from the moment of oviposition until they have reached the intestinal lumen. Half of the eggs does not succeed to exit the blood vessel. They are dragged along with the blood stream and get stuck in small vessels. Eggs elicit a strong immune response and those stuck in the host tissues hence induce granuloma formation.

While schistosome adult worms must prevent an effective immune response, eggs need the immune system in order to be excreted [49]. The schistosome eggshell and egg secretions contain many immunogenic proteins and glycans [30, 40, 110, 149] (**chapter 2**). These immunogenic components of eggshell and egg secretions may be of major interest in the scope of immune mediated egg excretion. Eggshells and egg secretions induce a Th2 response via IL-4 activated macrophages and CD4+ T-lymphocytes. Fecal excretion of eggs is immune mediated and egg excretion rate and CD4+ T-cell percentage are positively correlated [49, 90]. Both major egg secretion products IPSE/alpha-1 and Omega-1 alone are capable of Th2 skewing [59, 163, 164, 178], but secretion of these glycoproteins only starts a few days after oviposition when the egg has matured. Because maturation takes about a week, T-cells are likely to play a role during penetration through the gut wall, when the eggs have already adhered to and passed through the endothelium.

The schistosome eggshell contains more than specific eggshell proteins alone. The observed incorporation of these other proteins as reported in **chapter 2**, probably originate from neighbouring vitelline cells. This may appear as an unspecific

feature of eggshell production. But many of the proteins identified to be part of the eggshell are known schistosome antigens, such as p40, phosphoenolpyruvate carboxykinase (PEPCK) and thioredoxin peroxidase. These proteins induce cellular immune responses [3, 200] or antibody responses [122]. They may hence prime the immune system and contribute to the penetration of the eggs through host tissues.

However useful the immune response may be for extravasation, immune activation has a hindside. The main cause of pathology in schistosomiasis are the non-excreted eggs which are trapped in the host tissue, generally the liver. Trapped eggs are enveloped in granulomas which are composed of eosinophils, CD4+ T-lymphocytes, macrophages and collagen fibres. It is the Th2 response generated by the host against antigens secreted by the parasite eggs that is responsible for the granuloma formation [137]. In absence of the Th2 response, granulomas are not formed and continuous production of egg secretion products leads to severe immunopathology and hepatotoxic liver damage. In experimentally infected mice, this rapidly leads to death of the mice. Granulomas isolate the eggs and prevent the further spread of egg secretions. As the eggs die, the granulomas resolve, leaving fibrotic plaques. Accumulation of eggs and hence granulomas can eventually lead to permanent fibrotic changes in the liver, liver damage and portal hypertension.

In addition to the findings that eggshell contains a range of proteins known to be immunogenic, **chapter 3** shows that immune sera contain antibodies which react with the eggshell. The formed antigen-antibody complex can induce chronic inflammatory responses where the interaction between CD4+ T-cells, macrophages and cytokines can cause granulomas to form [201].

Next to proteins, eggshell contains glycans. Schistosome glycans are the major inducers of the immune response [188] and the synthetic glycans GalNAc β -4GlcNAc (LacdiNAc, LDN) and Gal β 1-4GlcNAc (LacNAc, LN) alone can induce granulomas [185].

Both eggshells and egg secretions induce antibody responses, as do adult worms. There is some overlap between worm and egg antigens, but there are enough differences to employ them usefully for diagnostics. That is described in **chapter 5**, where the differences in antibody responses to eggs and worms are used to differentiate between neuroschistosomiasis and other causes of transverse myelitis. It is essential to be certain of the diagnosis neuroschistosomiasis, before the start of therapy that includes immunosuppressive corticosteroids. These are contraindicated in case of viral or bacterial causes of transverse myelitis. The key feature

of the proposed test is to discriminate between antibodies in the cerebrospinal fluid (CSF) produced *in situ* and leakage of antibodies from blood plasma into the CSF due to damage to the blood-brain-barrier. The former indicates the presence of schistosome antigens in the CNS, while the latter points to another cause of the transverse myelitis. As neuroschistosomiasis is a result of nerve compression due to inflammation around schistosome eggs deposited near the nerves, the hypothesis is that anti-egg IgG, when normalized to anti-worm IgG, would be enriched in the CSF compared to the blood. Two schistosome-positive patients with transverse myelitis with an infectious cause for which no other pathogen was identified, had a positive test result, i.e. had a ratio >2 . The egg-worm antibody index appears to be a valuable tool in the confirmation of neuroschistosomiasis. Further validation of this ratio is required in a larger group of patients, both travelers and patients in schistosome-endemic areas. It is important to distinguish these two groups of patients, as in endemic regions antibody levels may drop below cut-off values in chronic or recurrent infections.

Worm-host interactions

Many parasitic worms travel through host tissues at some point in their life cycle. They are then in constant interaction with the host immune system and have to prevent a damaging immune response. The outer surface of the worms is a major site of host-parasite interaction. It protects the worms and has other functions involved in immune invasion, excretion of proteins and uptake of nutrients.

The surface of blood-dwelling trematodes is the tegument. It consists of two lipid bilayers that overlay the syncytium. It forms a large surface made of lipids, glycans and proteins which can be detected by the immune system. In addition, (immunomodulatory) schistosome proteins are secreted through the tegument.

The protein composition of the tegument has been studied extensively [15–17, 184]. These tegumental proteins consist of membrane proteins, cytoskeleton proteins, proteins and secreted proteins. There is also a number of proteins unique to schistosomes that were specifically detected in the tegument [184].

Lipids form a major component in tegument, which contains high amounts of cholesterol, sphingomyelin and saturated (ether-linked) phospholipid species [2, 151]. The most abundant phospholipid classes in membranes of schistosomes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The diacyl-phospholipid species composition has been characterized for these phospholipid

classes and this revealed that the species composition of these phospholipids is tegument-specific and differs from that of whole worm [21]. In **chapter 6** an analysis of the species composition of all major phospholipid classes is given, including phosphatidylinositol (PI), phosphatidylserine (PS) and lysophospholipids in isolated tegumental membranes. For most phospholipid classes, the species composition is substantially different in the tegument compared to whole worms. This is true for the PC and PS species. In contrast, diacyl-phospholipid species composition is strikingly similar for PI species. Furthermore, the tegument membranes were specifically enriched in lysophospholipids, especially lysophosphatidylserine (lyso-PS) and lysophosphatidylethanolamine (lyso-PE). In addition, the tegument was enriched in eicosaenoic acid (20:1) containing lysophospholipids in the phospholipid classes PS and PE, but not in PC and PI.

Lysophospholipids are minor compounds in membranes, but they play an important role in signal transduction. They also play a role in host-parasite interactions. It has been described that schistosome lysophosphatidylcholine (lyso-PC) can lead to lysis of red blood cells and cause immobilization of membrane components of red blood cells [71]. Lyso-PS species have been shown to activate TLR2 on host immune cells and induce the development of IL-10 producing regulatory T- cells [187]. Hence schistosome lyso-PS from the tegument may down regulate the immune response. Although tegument appeared to be specifically enriched in lyso-PS, no lyso-PS could be detected as a secreted phospholipid in incubation medium or in host plasma (**chapter 6**). Circulating amounts are thus below detection level. This may be because lysophospholipids do not bind to albumin. Due to their amphipathic nature, lysophospholipids readily incorporate into membranes. This is especially true for schistosome lysophospholipids with long chain fatty acids that are relatively hydrophobic. *In vitro*, no acceptor membrane is available for excreted lysophospholipids. Hence, lysophospholipids possibly reincorporate into the tegumental membranes as the only available phospholipid membranes. *In vivo*, the acceptor membranes could be those of endothelial cells or those from circulating cells. While no free circulating (tegument specific) lyso-PS could be detected in plasma, it may still be excreted and immediately come in contact with the effector cells of the immune system and activate them to induce a regulatory immune response. Furthermore, incorporation of schistosome (lyso)phospholipids into membranes of host immune effector cells or endothelial cells may alter the biophysical properties of the membranes because of the structure of these unusual long and unsaturated fatty acids.

Phospholipid analysis in blood plasma revealed an interesting difference between phospholipid composition in blood plasma of infected hamsters and that of non-infected hamsters. Blood plasma of hamsters infected with schistosomes had substantially lower PI content. These host phospholipids have precursor functions in lipid signalling, cell signalling and membrane trafficking. Reduction of PI by schistosomes seems peculiar and there are no mechanisms known to contribute to this.

Unlike fatty acids, phospholipids do not bind albumin. PI in blood plasma are likely to constitute exosomes or microvesicles. Exosomes are released by activated cells of the immune system and are involved in intercellular communication to mediate the activation of the immune response [179]. Reduction in PI may indicate that schistosomes alter the release of exosomes, which may affect the immune response.

7.4 Concluding remarks/future directives

Both schistosome worms and schistosome eggs are in constant interaction with their host. Worms and eggs have defined interactive surfaces which are highly unusual and specific in nature and composition. Further analysis of these structures could provide valuable information about the biology of schistosomes. Because these structures are so specific, they could serve as targets for vaccine and anti-schistosomal drug development.

References

1. Abath FG and Werkhauser RC. The tegument of *Schistosoma mansoni*: functional and immunological features. *Parasite Immunol*, **1996**. 18:15–20.
2. Allan D, Payares G and Evans WH. The phospholipid and fatty acid composition of *Schistosoma mansoni* and of its purified tegumental membranes. *Mol Biochem Parasitol*, **1987**. 23:123–128.
3. Asahi H, Hernandez HJ and Stadecker MJ. A novel 62-kilodalton egg antigen from *Schistosoma mansoni* induces a potent CD4(+) T helper cell response in the C57BL/6 mouse. *Infect Immun*, **1999**. 67:1729–1735.
4. Asahi H, Osman A, Cook RM, LoVerde PT and Stadecker MJ. *Schistosoma mansoni* phosphoenolpyruvate carboxykinase, a novel egg antigen: immunological properties of the recombinant protein and identification of a T-cell epitope. *Infect Immun*, **2000**. 68:3385–3393.
5. Asch HL and Read CP. Transtegumental absorption of amino acids by male *Schistosoma mansoni*. *J Parasitol*, **1975**. 61:378–379.
6. Ashton PD, Harrop R, Shah B and Wilson RA. The schistosome egg: development and secretions. *Parasitology*, **2001**. 122:329–338.
7. Barreto ML, Silva JT, Mott KE and Lehman JS. Stability of faecal egg excretion in *Schistosoma mansoni* infection. *Trans R Soc Trop Med Hyg*, **1978**. 72:181–187.
8. Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S and Marcilla A. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. *FEBS Lett*, **2004**. 563:203–206.
9. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, Mashiyama ST, Al-Lazikani B, Andrade LF, Ashton PD, Aslett MA, Bartholomeu DC, Blandin G, Caffrey CR, Coghlan A, Coulson R, Day TA, Delcher A, DeMarco R, Djikeng A, Eyre T, Gamble JA, Ghedin E, Gu Y, Hertz-Fowler C, Hirai H, Hirai Y, Houston R, Ivens A, Johnston DA, Lacerda D, Macedo CD, McVeigh P, Ning Z, Oliveira G, Overington JP, Parkhill J, Pertea M, Pierce RJ, Protasio AV, Quail MA, Rajandream MA, Rogers J, Sajid M, Salzberg SL, Stanke M, Tivey AR, White O, Williams DL, Wortman J, Wu W, Zamanian M, Zerlotini A, Fraser-Liggett CM, Barrell BG and El-Sayed NM. The genome of the blood fluke *Schistosoma mansoni*. *Nature*, **2009**. 460:352–358.
10. Bhowmick IP, Kumar N, Sharma S, Coppens I and Jarori GK. *Plasmodium falciparum* enolase: stage-specific expression and sub-cellular localization. *Malar J*, **2009**. 8:179.

11. Bittner S. When quinones meet amino acids: chemical, physical and biological consequences. *Amino Acids*, **2006**. 30:205–224.
12. Bligh EG and Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*, **1959**. 37:911–917.
13. Bobek L, Rekosh DM, van Keulen H and LoVerde PT. Characterization of a female-specific cDNA derived from a developmentally regulated mRNA in the human blood fluke *Schistosoma mansoni*. *Proc Natl Acad Sci U S A*, **1986**. 83:5544–5548.
14. Bobek LA, Rekosh DM and LoVerde PT. Small gene family encoding an eggshell (chorion) protein of the human parasite *Schistosoma mansoni*. *Mol Cell Biol*, **1988**. 8:3008–3016.
15. Braschi S, Borges WC and Wilson RA. Proteomic analysis of the schistosome tegument and its surface membranes. *Mem Inst Oswaldo Cruz*, **2006**. 101 Suppl 1:205–212.
16. Braschi S, Curwen RS, Ashton PD, Verjovski-Almeida S and Wilson A. The tegument surface membranes of the human blood parasite *Schistosoma mansoni*: a proteomic analysis after differential extraction. *Proteomics*, **2006**. 6:1471–1482.
17. Braschi S and Wilson RA. Proteins exposed at the adult schistosome surface revealed by biotinylation. *Mol Cell Proteomics*, **2006**. 5:347–356.
18. Brouwers JFHM, Gadella BM, van Golde LM and Tielens AGM. Quantitative analysis of phosphatidylcholine molecular species using HPLC and light scattering detection. *J Lipid Res*, **1998**. 39:344–353.
19. Brouwers JFHM, Skelly PJ, Van Golde LM and Tielens AGM. Studies on phospholipid turnover argue against sloughing of tegumental membranes in adult *Schistosoma mansoni*. *Parasitology*, **1999**. 119:287–294.
20. Brouwers JFHM, Smeenk IM, van Golde LM and Tielens AGM. The incorporation, modification and turnover of fatty acids in adult *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1997**. 88:175–185.
21. Brouwers JFHM, van Hellemond JJ, van Golde LM and Tielens AGM. Ether lipids and their possible physiological function in adult *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1998**. 96:49–58.
22. Brouwers JFHM, Versluis C, van Golde LM and Tielens AGM. 5-octadecenoic acid: evidence for a novel type of fatty acid modification in schistosomes. *Biochem J*, **1998**. 334:315–319.
23. Brügger B, Erben G, Sandhoff R, Wieland FT and Lehmann WD. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *Proc Natl Acad Sci U S A*, **1997**. 94:2339–2344.
24. Byram JE and Senft AW. Structure of the schistosome eggshell: amino acid analysis and incorporation of labelled amino acids. *Am J Trop Med Hyg*, **1979**. 28:539–547.
25. Camacho M and Agnew A. *Schistosoma*: rate of glucose import is altered by acetylcholine interaction with tegumental acetylcholine receptors and acetylcholinesterase. *Exp Parasitol*, **1995**. 81:584–591.
26. Carod-Artal FJ. Neurological complications of *Schistosoma* infection. *Trans R Soc Trop Med Hyg*, **2008**. 102:107–116.
27. Carod-Artal FJ. Neuroschistosomiasis. *Expert Rev Anti Infect Ther*, **2010**. 8:1307–1318.

28. Carod-Artal FJ. Cerebral and spinal schistosomiasis. *Curr Neurol Neurosci Rep*, **2012**. 12:666–674.
29. Carod-Artal FJ, Vargas AP, Horan TA, Marinho PB and Coelho Costa PH. *Schistosoma mansoni* myelopathy: clinical and pathologic findings. *Neurology*, **2004**. 63:388–391.
30. Cass CL, Johnson JR, Califf LL, Xu T, Hernandez HJ, Stadecker MJ, Yates JR and Williams DL. Proteomic analysis of *Schistosoma mansoni* egg secretions. *Mol Biochem Parasitol*, **2007**. 155:84–93.
31. Castro-Borges W, Simpson DM, Dowle A, Curwen RS, Thomas-Oates J, Beynon RJ and Wilson RA. Abundance of tegument surface proteins in the human blood fluke *Schistosoma mansoni* determined by QconCAT proteomics. *J Proteomics*, **2011**. 74:1519–1533.
32. Cheever AW, Macedonia JG, Mosimann JE and Cheever EA. Kinetics of egg production and egg excretion by *Schistosoma mansoni* and *S. japonicum* in mice infected with a single pair of worms. *Am J Trop Med Hyg*, **1994**. 50:281–295.
33. Chen LL, Rekosh DM and LoVerde PT. *Schistosoma mansoni* p48 eggshell protein gene: characterization, developmentally regulated expression and comparison to the p14 eggshell protein gene. *Mol Biochem Parasitol*, **1992**. 52:39–52.
34. Chiang CP and Caulfield JP. The binding of human low-density lipoproteins to the surface of schistosomula of *Schistosoma mansoni* is inhibited by polyanions and reduces the binding of anti-schistosomal antibodies. *Am J Pathol*, **1989**. 134:1007–1018.
35. Clerinx J and Gompel AV. Schistosomiasis in travellers and migrants. *Travel Med Infect Dis*, **2011**. 9:6–24.
36. Cnops L, Soentjens P, Clerinx J and Esbroeck MV. A *Schistosoma haematobium*-specific real-time PCR for diagnosis of urogenital schistosomiasis in serum samples of international travelers and migrants. *PLoS Negl Trop Dis*, **2013**. 7:e2413.
37. Colley DG, Bustinduy AL, Secor WE and King CH. Human schistosomiasis. *Lancet*, **2014**. 383:2253–2264.
38. Cordingley JS, Taylor DW, Dunne DW and Butterworth AE. Clone banks of cDNA from the parasite *Schistosoma mansoni*: isolation of clones containing a potentially immunodiagnostic antigen gene. *Gene*, **1983**. 26:25–39.
39. Cornford EM. *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*: permeability to acidic amino acids and effect of separated and unseparated adults. *Exp Parasitol*, **1985**. 59:355–363.
40. Curwen RS, Ashton PD, Johnston DA and Wilson RA. The *Schistosoma mansoni* soluble proteome: a comparison across four life-cycle stages. *Mol Biochem Parasitol*, **2004**. 138:57–66.
41. de Boer AR, Hokke CH, Deelder AM and Wuhrer M. General microarray technique for immobilization and screening of natural glycans. *Anal Chem*, **2007**. 79:8107–8113.
42. de Jongste AH, Tilanus AM, Bax H, Willems MH, van der Feltz M and van Hellemond JJ. New insights in diagnosing *Schistosoma* myelopathy. *J Infect*, **2010**. 60:244–247.
43. de la Torre-Escudero E, Manzano-Román R, Pérez-Sánchez R, Siles-Lucas M and Oleaga A. Cloning and characterization of a plasminogen-binding surface-associated enolase from *Schistosoma bovis*. *Vet Parasitol*, **2010**. 173:76–84.

44. de Vlas SJ and Gryseels B. Underestimation of *Schistosoma mansoni* prevalences. *Parasitol Today*, **1992**. 8:274–277.
45. de Vlas SJ, Gryseels B, Oortmarssen GJV, Polderman AM and Habbema JD. A model for variations in single and repeated egg counts in *Schistosoma mansoni* infections. *Parasitology*, **1992**. 104:451–460.
46. Deelder AM, van Dam GJ, Kornelis D, Fillié YE and van Zeyl RJ. *Schistosoma*: analysis of monoclonal antibodies reactive with the circulating antigens CAA and CCA. *Parasitology*, **1996**. 112:21–35.
47. deWalick S, Bexkens ML, van Balkom BWM, Wu YP, Smit CH, Hokke CH, de Groot PG, Heck AJR, Tielens AGM and van Hellemond JJ. The proteome of the insoluble *Schistosoma mansoni* eggshell skeleton. *Int J Parasitol*, **2011**. 41:525–532.
48. deWalick S, Tielens AGM and van Hellemond JJ. *Schistosoma mansoni*: the egg, biosynthesis of the shell and interaction with the host. *Exp Parasitol*, **2012**. 132:7–13.
49. Doenhoff M, Musallam R, Bain J and McGregor A. Studies on the host-parasite relationship in *Schistosoma mansoni*-infected mice: the immunological dependence of parasite egg excretion. *Immunology*, **1978**. 35:771–778.
50. Doenhoff MJ, Cioli D and Utzinger J. Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. *Curr Opin Infect Dis*, **2008**. 21:659–667.
51. Doenhoff MJ, Stanley RG, Pryce D, Curtis RCH, Parry H, Griffiths K and Jackson CL. Identification of a fibrinolytic enzyme in *Schistosoma mansoni* eggs and modulated blood fibrinogen metabolism in *S. mansoni*-infected mice. *Parasitology*, **2003**. 126:231–234.
52. Donnelly S, Stack CM, O'Neill SM, Sayed AA, Williams DL and Dalton JP. Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. *FASEB J*, **2008**. 22:4022–4032.
53. Dunne DW and Cooke A. A worm's eye view of the immune system: consequences for evolution of human autoimmune disease. *Nat Rev Immunol*, **2005**. 5:420–426.
54. Dunne DW, Hassounah O, Musallam R, Lucas S, Pepys MB, Baltz M and Doenhoff M. Mechanisms of *Schistosoma mansoni* egg excretion: parasitological observations in immunosuppressed mice reconstituted with immune serum. *Parasite Immunol*, **1983**. 5:47–60.
55. El-Awady MK, Gad YZ, Wen Y, Eassawi M, Effat L, Amr KS, Ismail S and Christ GJ. *Schistosoma hematobium* soluble egg antigens induce proliferation of urothelial and endothelial cells. *World J Urol*, **2001**. 19:263–266.
56. Erasmus DA. The subcellular localization of labelled tyrosine in the vitelline cells of *Schistosoma mansoni*. *Z Parasitenkd*, **1975**. 46:75–81.
57. Esgleas M, Li Y, Hancock MA, Harel J, Dubreuil JD and Gottschalk M. Isolation and characterization of alpha-enolase, a novel fibronectin-binding protein from *Streptococcus suis*. *Microbiology*, **2008**. 154:2668–2679.
58. Esterre P, Raobelison A, Ramarokoto CE, Ravaoalimalala VE, Boisier P and Roux J. Serum concentrations of sICAM-1, sE-, sP- and sL-selectins in patients with *Schistosoma mansoni* infection and association with disease severity. *Parasite Immunol*, **1998**. 20:369–376.

59. Everts B, Perona-Wright G, Smits HH, Hokke CH, van der Ham AJ, Fitzsimmons CM, Doenhoff MJ, van der Bosch J, Mohrs K, Haas H, Mohrs M, Yazdanbakhsh M and Schramm G. Omega-1, a glycoprotein secreted by *Schistosoma mansoni* eggs, drives Th2 responses. *J Exp Med*, **2009**. 206:1673–1680.
60. Ferrari TC, Correa-Oliveira R, Xavier MA, Gazzinelli G and Cunha AS. Estimation of the local synthesis of immunoglobulin G (IgG) in the central nervous system of patients with spinal cord schistosomiasis by the IgG index. *Trans R Soc Trop Med Hyg*, **1999**. 93:558–559.
61. Ferrari TC, Moreira PR, Oliveira RC, Ferrari ML, Gazzinelli G and Cunha AS. The value of an enzyme-linked immunosorbent assay for the diagnosis of schistosomiasis mansoni myeloradiculopathy. *Trans R Soc Trop Med Hyg*, **1995**. 89:496–500.
62. Ferrari TCA and Moreira PRR. Neuroschistosomiasis: clinical symptoms and pathogenesis. *Lancet Neurol*, **2011**. 10:853–864.
63. Ferrari TCA, Moreira PRR and Cunha AS. Clinical characterization of neuroschistosomiasis due to *Schistosoma mansoni* and its treatment. *Acta Trop*, **2008**. 108:89–97.
64. File S. Interaction of schistosome eggs with vascular endothelium. *J Parasitol*, **1995**. 81:234–238.
65. Fitzpatrick JM, Hirai Y, Hirai H and Hoffmann KF. Schistosome egg production is dependent upon the activities of two developmentally regulated tyrosinases. *FASEB J*, **2007**. 21:823–835.
66. Ford JW, Hillyer GV and Connelly TG. The circumoval precipitin reaction on *Schistosoma mansoni* eggs as seen by scanning electron microscopy. *Am J Trop Med Hyg*, **1980**. 29:1246–1248.
67. Freedman DO and Ottesen EA. Eggs of *Schistosoma mansoni* stimulate endothelial cell proliferation *in vitro*. *J Infect Dis*, **1988**. 158:556–562.
68. Fripp PJ, Williams G and Crawford MA. The differences between the long chain polyenoic acids of adult *Schistosoma mansoni* and the serum of its host. *Comp Biochem Physiol B*, **1976**. 53:505–507.
69. Furlong ST and Caulfield JP. *Schistosoma mansoni*: synthesis and release of phospholipids, lysophospholipids, and neutral lipids by schistosomula. *Exp Parasitol*, **1989**. 69:65–77.
70. Gobert GN, Stenzel DJ, McManus DP and Jones MK. The ultrastructural architecture of the adult *Schistosoma japonicum* tegument. *Int J Parasitol*, **2003**. 33:1561–1575.
71. Golan DE, Brown CS, Cianci CM, Furlong ST and Caulfield JP. Schistosomula of *Schistosoma mansoni* use lysophosphatidylcholine to lyse adherent human red blood cells and immobilize red cell membrane components. *J Cell Biol*, **1986**. 103:819–828.
72. Gönner R. [Schistosomiasis studies. II. Oogenesis of *Schistosoma mansoni* and the development of the eggs in the host organism]. *Z Tropenmed Parasitol*, **1955**. 6:33–52.
73. Grandière-Pérez L, Ansart S, Paris L, Faussart A, Jauréguiberry S, Grivois JP, Klement E, Bricaire F, Danis M and Caumes E. Efficacy of praziquantel during the incubation and invasive phase of *Schistosoma haematobium* schistosomiasis in 18 travelers. *Am J Trop Med Hyg*, **2006**. 74:814–818.
74. Gryseels B. Schistosomiasis. *Infect Dis Clin North Am*, **2012**. 26:383–397.
75. Gryseels B, Polman K, Clerinx J and Kestens L. Human schistosomiasis. *Lancet*, **2006**. 368:1106–1118.

76. Grzych JM, Pearce E, Cheever A, Caulada ZA, Caspar P, Heiny S, Lewis F and Sher A. Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni. *J Immunol*, **1991**. 146:1322–1327.
77. Guillou F, Roger E, Moné Y, Rognon A, Grunau C, Théron A, Mitta G, Coustau C and Gourbal BEF. Excretory-secretory proteome of larval *Schistosoma mansoni* and *Echinostoma caproni*, two parasites of *Biomphalaria glabrata*. *Mol Biochem Parasitol*, **2007**. 155:45–56.
78. Hagan P. Reinfection, exposure and immunity in human schistosomiasis. *Parasitol Today*, **1992**. 8:12–16.
79. Han ZG, Brindley PJ, Wang SY and Chen Z. *Schistosoma* genomics: new perspectives on schistosome biology and host-parasite interaction. *Annu Rev Genomics Hum Genet*, **2009**. 10:211–240.
80. Herbert DR, Orekov T, Perkins C and Finkelman FD. IL-10 and TGF-beta redundantly protect against severe liver injury and mortality during acute schistosomiasis. *J Immunol*, **2008**. 181:7214–7220.
81. Hockley DJ. Ultrastructure of the tegument of *Schistosoma*. *Adv Parasitol*, **1973**. 11:233–305.
82. Hockley DJ and McLaren DJ. *Schistosoma mansoni*: changes in the outer membrane of the tegument during development from cercaria to adult worm. *Int J Parasitol*, **1973**. 3:13–25.
83. Hockley DJ, McLaren DJ, Ward BJ and Nermut MV. A freeze-fracture study of the tegumental membrane of *Schistosoma mansoni* (platyhelminthes:trematoda). *Tissue Cell*, **1975**. 7:485–496.
84. Hoffmann KF, Cheever AW and Wynn TA. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol*, **2000**. 164:6406–6416.
85. Hokke CH, Deelder AM, Hoffmann KF and Wuhrer M. Glycomics-driven discoveries in schistosome research. *Exp Parasitol*, **2007**. 117:275–283.
86. Hudson PJ, Dobson AP and Lafferty KD. Is a healthy ecosystem one that is rich in parasites? *Trends Ecol Evol*, **2006**. 21:381–385.
87. Jauréguiberry S, Ansart S, Perez L, Danis M, Bricaire F and Caumes E. Acute neuroschistosomiasis: two cases associated with cerebral vasculitis. *Am J Trop Med Hyg*, **2007**. 76:964–966.
88. Johnson KS, Taylor DW and Cordingley JS. Possible eggshell protein gene from *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1987**. 22:89–100.
89. Jourdane J and Théron A. Larval development: eggs to cercariae. In: Rollinson D and Simpson AJG, eds., *The biology of schistosomes, from genes to latrines*, 83–113. Academic Press, London, **1987**.
90. Karanja DM, Colley DG, Nahlen BL, Ouma JH and Secor WE. Studies on schistosomiasis in western Kenya: I. evidence for immune-facilitated excretion of schistosome eggs from patients with *Schistosoma mansoni* and human immunodeficiency virus coinfections. *Am J Trop Med Hyg*, **1997**. 56:515–521.
91. Katz N, Chaves A and Pellegrino J. A simple device for quantitative stool thick-smear technique in *Schistosomiasis mansoni*. *Rev Inst Med Trop Sao Paulo*, **1972**. 14:397–400.
92. Keller A, Nesvizhskii AI, Kolker E and Aebersold R. Empirical statistical model to estimate

- the accuracy of peptide identifications made by ms/ms and database search. *Anal Chem*, **2002**. 74:5383–5392.
93. Köster B, Dargatz H, Schröder J, Hirzmann J, Haarmann C, Symmons P and Kunz W. Identification and localisation of the products of a putative eggshell precursor gene in the vitellarium of *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1988**. 31:183–198.
94. Kunz W, Opatz K, Finken M and Symmons P. Sequences of two genomic fragments containing an identical coding region for a putative egg-shell precursor protein of *Schistosoma mansoni*. *Nucleic Acids Res*, **1987**. 15:5894.
95. Lankhof H, Damas C, Schiphorst ME, IJsseldijk MJ, Bracke M, Furlan M, Tsai HM, de Groot PG, Sixma JJ and Vink T. von Willebrand factor without the A2 domain is resistant to proteolysis. *Thromb Haemost*, **1997**. 77:1008–1013.
96. Lankhof H, van Hoeij M, Schiphorst ME, Bracke M, Wu YP, IJsseldijk MJ, Vink T, de Groot PG and Sixma JJ. A3 domain is essential for interaction of von Willebrand factor with collagen type III. *Thromb Haemost*, **1996**. 75:950–958.
97. Lankhof H, Wu YP, Vink T, Schiphorst ME, Zerwes HG, de Groot PG and Sixma JJ. Role of the glycoprotein Ib-binding A1 repeat and the RGD sequence in platelet adhesion to human recombinant von Willebrand factor. *Blood*, **1995**. 86:1035–1042.
98. Lejoly-Boisseau H, Appriou M, Seigneur M, Pruvost A, Tribouley-Duret J and Tribouley J. *Schistosoma mansoni*: *in vitro* adhesion of parasite eggs to the vascular endothelium. subsequent inhibition by a monoclonal antibody directed to a carbohydrate epitope. *Exp Parasitol*, **1999**. 91:20–29.
99. Li Y, Yu DB, Li YS, Luo XS, Liang YS, Bartley PB and McManus DP. Antibody isotype responses to *Schistosoma japonicum* antigens in subjects from a schistosomiasis area with repeated praziquantel chemotherapy compared with a new endemic zone in Hunan Province, P.R. China. *Trans R Soc Trop Med Hyg*, **2002**. 96:210–215.
100. Liu F, Lu J, Hu W, Wang SY, Cui SJ, Chi M, Yan Q, Wang XR, Song HD, Xu XN, Wang JJ, Zhang XL, Zhang X, Wang ZQ, Xue CL, Brindley PJ, McManus DP, Yang PY, Feng Z, Chen Z and Han ZG. New perspectives on host-parasite interplay by comparative transcriptomic and proteomic analyses of *Schistosoma japonicum*. *PLoS Pathog*, **2006**. 2:e29.
101. Long GW, Yogore MG, Lewert RM, Blas BL and Pelley RP. Efficacy of purified *Schistosoma japonicum* egg antigens for ELISA serodiagnosis of human schistosomiasis japonica: specificity and sensitivity. *Am J Trop Med Hyg*, **1982**. 31:1006–1014.
102. Loverde PT and Chen L. Schistosome female reproductive development. *Parasitol Today*, **1991**. 7:303–308.
103. Lowe GDO. Virchow's triad revisited: abnormal flow. *Pathophysiol Haemost Thromb*, **2003**. 33:455–457.
104. Luger AF, Schmidt BL and Kaulich M. Significance of laboratory findings for the diagnosis of neurosyphilis. *Int J STD AIDS*, **2000**. 11:224–234.
105. Luo GP, Ni B, Yang X and Wu YZ. von Willebrand factor: more than a regulator of hemostasis and thrombosis. *Acta Haematol*, **2012**. 128:158–169.

106. Magalhães KG, Almeida PE, Atella GC, Maya-Monteiro CM, Castro-Faria-Neto HC, Pelajo-Machado M, Lenzi HL, Bozza MT and Bozza PT. Schistosomal-derived lysophosphatidylcholine are involved in eosinophil activation and recruitment through Toll-like receptor-2-dependent mechanisms. *J Infect Dis*, **2010**. 202:1369–1379.
107. Magalhães-Santos IF, Lemaire DC, Andrade-Filho AS, Queiroz AC, Carvalho OM, Carmo TMA, Siqueira IC, Andrade DM, Rego MF, Guedes APT and Reis MG. Antibodies to *Schistosoma mansoni* in human cerebrospinal fluid. *Am J Trop Med Hyg*, **2003**. 68:294–298.
108. Maizels RM. Infections and allergy - helminths, hygiene and host immune regulation. *Curr Opin Immunol*, **2005**. 17:656–661.
109. Mathieson W, Castro-Borges W and Wilson RA. The proteasome-ubiquitin pathway in the *Schistosoma mansoni* egg has development- and morphology-specific characteristics. *Mol Biochem Parasitol*, **2011**. 175:118–125.
110. Mathieson W and Wilson RA. A comparative proteomic study of the undeveloped and developed *Schistosoma mansoni* egg and its contents: the miracidium, hatch fluid and secretions. *Int J Parasitol*, **2010**. 40:617–628.
111. McLaren DJ. Disguise as an evasive stratagem of parasitic organisms. *Parasitology*, **1984**. 88:597–611.
112. McLaren DJ, Clegg JA and Smithers SR. Acquisition of host antigens by young *Schistosoma mansoni* in mice: correlation with failure to bind antibody *in vitro*. *Parasitology*, **1975**. 70:67–75.
113. McLaren DJ and Hockley DJ. Blood flukes have a double outer membrane. *Nature*, **1977**. 269:147–149.
114. McManus DP and Loukas A. Current status of vaccines for schistosomiasis. *Clin Microbiol Rev*, **2008**. 21:225–242.
115. Mebius MM, van Genderen PJJ, Urbanus RT, Tielens AGM, de Groot PG and van Hellemond JJ. Interference with the host haemostatic system by schistosomes. *PLoS Pathog*, **2013**. 9:e1003781.
116. Meiring HD, van der Heeft E, ten Hove GJ and de Jong APJM. Nanoscale LC-MS(n): technical design and applications to peptide and protein analysis. *Journal of Separation Science*, **2002**. 25:557–568.
117. Menrath M, Michel A and Kunz W. A female-specific cDNA sequence of *Schistosoma mansoni* encoding a mucin-like protein that is expressed in the epithelial cells of the reproductive duct. *Parasitology*, **1995**. 111:477–483.
118. Meyer F, Meyer H and Bueding E. Lipid metabolism in the parasitic and free-living flatworms, *Schistosoma mansoni* and *Dugesia dorotocephala*. *Biochim Biophys Acta*, **1970**. 210:257–266.
119. Michel A, Knobloch J and Kunz W. P19: a female and tissue specifically expressed gene in *Schistosoma mansoni*, regulated by pairing with the male. *Parasitology*, **2003**. 127:519–524.
120. Moffat D and Kusel JR. Fluorescent lipid uptake and transport in adult *Schistosoma mansoni*. *Parasitology*, **1992**. 105:81–89.
121. Moser D, Doumbo O and Klinkert MQ. The humoral response to heat shock protein 70 in human and murine schistosomiasis mansoni. *Parasite Immunol*, **1990**. 12:341–352.

122. Mutapi F, Burchmore R, Mduluzza T, Foucher A, Harcus Y, Nicoll G, Midzi N, Turner CM and Maizels RM. Praziquantel treatment of individuals exposed to *Schistosoma haematobium* enhances serological recognition of defined parasite antigens. *J Infect Dis*, **2005**. 192:1108–1118.
123. Neill PJ, Smith JH, Doughty BL and Kemp M. The ultrastructure of the *Schistosoma mansoni* egg. *Am J Trop Med Hyg*, **1988**. 39:52–65.
124. Nesvizhskii AI, Keller A, Kolker E and Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem*, **2003**. 75:4646–4658.
125. Ngaiza JR and Doenhoff MJ. Blood platelets and schistosome egg excretion. *Proc Soc Exp Biol Med*, **1990**. 193:73–79.
126. Ngaiza JR, Doenhoff MJ and Jaffe EA. *Schistosoma mansoni* egg attachment to cultured human umbilical vein endothelial cells: an *in vitro* model of an early step of parasite egg excretion. *J Infect Dis*, **1993**. 168:1576–1580.
127. Obeng BB, Aryeetey YA, de Dood CJ, Amoah AS, Larbi IA, Deelder AM, Yazdanbakhsh M, Hartgers FC, Boakye DA, Verweij JJ, van Dam GJ and van Lieshout L. Application of a circulating-cathodic-antigen (CCA) strip test and real-time PCR, in comparison with microscopy, for the detection of *Schistosoma haematobium* in urine samples from Ghana. *Ann Trop Med Parasitol*, **2008**. 102:625–633.
128. Ojopi EPB, Oliveira PSL, Nunes DN, Paquola A, DeMarco R, Gregório SP, Aires KA, Menck CFM, Leite LCC, Verjovski-Almeida S and Dias-Neto E. A quantitative view of the transcriptome of *Schistosoma mansoni* adult-worms using SAGE. *BMC Genomics*, **2007**. 8:186.
129. Okano M, Satoskar AR, Nishizaki K, Abe M and Harn DA. Induction of Th2 responses and IgE is largely due to carbohydrates functioning as adjuvants on *Schistosoma mansoni* egg antigens. *J Immunol*, **1999**. 163:6712–6717.
130. Oliveira SDS, Quintas LEM, Amaral LS, Noël F, Farsky SH and Silva CLM. Increased endothelial cell-leukocyte interaction in murine schistosomiasis: possible priming of endothelial cells by the disease. *PLoS One*, **2011**. 6:e23547.
131. Pammenter MD, Haribhai HC, Epstein SR, Rossouw EJ, Bhigjee AI and Bill PL. The value of immunological approaches to the diagnosis of schistosomal myelopathy. *Am J Trop Med Hyg*, **1991**. 44:329–335.
132. Pancholi V and Chhatwal GS. Housekeeping enzymes as virulence factors for pathogens. *Int J Med Microbiol*, **2003**. 293:391–401.
133. Pearce EJ. Progress towards a vaccine for schistosomiasis. *Acta Trop*, **2003**. 86:309–313.
134. Pearce EJ. Priming of the immune response by schistosome eggs. *Parasite Immunol*, **2005**. 27:265–270.
135. Pearce EJ, Caspar P, Grzych JM, Lewis FA and Sher A. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J Exp Med*, **1991**. 173:159–166.
136. Pearce EJ, Cheever A, Leonard S, Covalesky M, Fernandez-Botran R, Kohler G and Kopf M. *Schistosoma mansoni* in IL-4-deficient mice. *Int Immunol*, **1996**. 8:435–444.

137. Pearce EJ and MacDonald AS. The immunobiology of schistosomiasis. *Nat Rev Immunol*, **2002**. 2:499–511.
138. Pino-Heiss S, Brown M and McKerrow JH. *Schistosoma mansoni*: degradation of host extracellular matrix by eggs and miracidia. *Exp Parasitol*, **1985**. 59:217–221.
139. Poulin R. The many roads to parasitism: a tale of convergence. *Adv Parasitol*, **2011**. 74:1–40.
140. Race GJ, Martin JH, Moore DV and Larsh JE. Scanning and transmission electronmicroscopy of *Schistosoma mansoni* eggs, cercariae, and adults. *Am J Trop Med Hyg*, **1971**. 20:914–924.
141. Ramajo-Hernández A, Pérez-Sánchez R, Ramajo-Martín V and Oleaga A. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. *Exp Parasitol*, **2007**. 115:83–91.
142. Reiber H. The discrimination between different blood-CSF barrier dysfunctions and inflammatory reactions of the CNS by a recent evaluation graph for the protein profile of cerebrospinal fluid. *J Neurol*, **1980**. 224:89–99.
143. Reiber H and Lange P. Quantification of virus-specific antibodies in cerebrospinal fluid and serum: sensitive and specific detection of antibody synthesis in brain. *Clin Chem*, **1991**. 37:1153–1160.
144. Reis MG, Kuhns J, Blanton R and Davis AH. Localization and pattern of expression of a female specific mRNA in *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1989**. 32:113–119.
145. Retra K, Bleijerveld OB, van Gestel RA, Tielens AGM, van Hellemond JJ and Brouwers JFHM. A simple and universal method for the separation and identification of phospholipid molecular species. *Rapid Commun Mass Spectrom*, **2008**. 22:1853–1862.
146. Retra K, van Riet E, Adegnikaa AA, Everts B, van Geest S, Kreamsner PG, van Hellemond JJ, van der Kleij D, Tielens AGM and Yazdanbakhsh M. Immunologic activity of schistosomal and bacterial TLR2 ligands in Gabonese children. *Parasite Immunol*, **2008**. 30:39–46.
147. Roberts SM, Aitken R, Vojvodic M, Wells E and Wilson RA. Identification of exposed components on the surface of adult *Schistosoma mansoni* by lactoperoxidase-catalysed iodination. *Mol Biochem Parasitol*, **1983**. 9:129–143.
148. Robijn MLM, Koeleman CAM, Wuhrer M, Royle L, Geyer R, Dwek RA, Rudd PM, Deelder AM and Hokke CH. Targeted identification of a unique glycan epitope of *Schistosoma mansoni* egg antigens using a diagnostic antibody. *Mol Biochem Parasitol*, **2007**. 151:148–161.
149. Robijn MLM, Wuhrer M, Kornelis D, Deelder AM, Geyer R and Hokke CH. Mapping fucosylated epitopes on glycoproteins and glycolipids of *Schistosoma mansoni* cercariae, adult worms and eggs. *Parasitology*, **2005**. 130:67–77.
150. Rodrigues V, Chaudhri M, Knight M, Meadows H, Chambers AE, Taylor WR, Kelly C and Simpson AJ. Predicted structure of a major *Schistosoma mansoni* eggshell protein. *Mol Biochem Parasitol*, **1989**. 32:7–13.
151. Rogers MV and McLaren DJ. Analysis of total and surface membrane lipids of *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1987**. 22:273–288.
152. Rogers RA, Jack RM and Furlong ST. Lipid and membrane protein transfer from human neutrophils to schistosomes is mediated by ligand binding. *J Cell Sci*, **1993**. 106:485–491.

153. Rogers SH and Bueding E. Anatomical localization of glucose uptake by *Schistosoma mansoni* adults. *Int J Parasitol*, **1975**. 5:369–371.
154. Ross AG, McManus DP, Farrar J, Hunstman RJ, Gray DJ and Li YS. Neuroschistosomiasis. *J Neurol*, **2012**. 259:22–32.
155. Ross AGP, Bartley PB, Sleigh AC, Olds GR, Li Y, Williams GM and McManus DP. Schistosomiasis. *N Engl J Med*, **2002**. 346:1212–1220.
156. Ruggeri ZM. Von Willebrand factor. *Curr Opin Hematol*, **2003**. 10:142–149.
157. Ruhaak LR, Steenvoorden E, Koeleman CAM, Deelder AM and Wuhrer M. 2-picoline-borane: a non-toxic reducing agent for oligosaccharide labeling by reductive amination. *Proteomics*, **2010**. 10:2330–2336.
158. Rumjanek FD, Campos EG and Afonso LC. Evidence for the occurrence of LDL receptors in extracts of schistosomula of *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1988**. 28:145–152.
159. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem*, **1998**. 67:395–424.
160. Sakamoto K and Ishii Y. Fine structure of schistosome eggs as seen through the scanning electron microscope. *Am J Trop Med Hyg*, **1976**. 25:841–844.
161. Sambrook J, Fritsch E and Maniatis T. *Molecular Cloning, a laboratory manual, 2nd ed*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, **1989**.
162. Schramm G, Gronow A, Knobloch J, Wippersteg V, Grevelding CG, Galle J, Fuller H, Stanley RG, Chiodini PL, Haas H and Doenhoff MJ. Ipse/alpha-1: a major immunogenic component secreted from *Schistosoma mansoni* eggs. *Mol Biochem Parasitol*, **2006**. 147:9–19.
163. Schramm G, Hamilton JV, Balog CIA, Wuhrer M, Gronow A, Beckmann S, Wippersteg V, Grevelding CG, Goldmann T, Weber E, Brattig NW, Deelder AM, Dunne DW, Hokke CH, Haas H and Doenhoff MJ. Molecular characterisation of kappa-5, a major antigenic glycoprotein from *Schistosoma mansoni* eggs. *Mol Biochem Parasitol*, **2009**. 166:4–14.
164. Schramm G, Mohrs K, Wodrich M, Doenhoff MJ, Pearce EJ, Haas H and Mohrs M. Cutting edge: Ipse/alpha-1, a glycoprotein from *Schistosoma mansoni* eggs, induces IgE-dependent, antigen-independent IL-4 production by murine basophils *in vivo*. *J Immunol*, **2007**. 178:6023–6027.
165. Seed JL and Bennett JL. *Schistosoma mansoni*: phenol oxidase's role in eggshell formation. *Exp Parasitol*, **1980**. 49:430–441.
166. Seglen PO. Preparation of isolated rat liver cells. *Methods Cell Biol*, **1976**. 13:29–83.
167. Sher A, Coffman RL, Hieny S, Scott P and Cheever AW. Interleukin 5 is required for the blood and tissue eosinophilia but not granuloma formation induced by infection with *Schistosoma mansoni*. *Proc Natl Acad Sci U S A*, **1990**. 87:61–65.
168. Silva CL, Morel N and Noël F. Portal veins of mice infected with *Schistosoma mansoni* exhibit an increased reactivity to 5-hydroxytryptamine. *Mem Inst Oswaldo Cruz*, **1998**. 93 Suppl 1:153–155.
169. Simpson AJ, Chaudri M, Knight M, Kelly C, Rumjanek F, Martin S and Smithers SR. Characterisation of the structure and expression of the gene encoding a major female specific polypeptide of *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1987**. 22:169–176.

170. Simpson AJ and Knight M. Cloning of a major developmentally regulated gene expressed in mature females of *Schistosoma mansoni*. *Mol Biochem Parasitol*, **1986**. 18:25–35.
171. Skelly PJ and Wilson RA. Making sense of the schistosome surface. *Adv Parasitol*, **2006**. 63:185–284.
172. Smith MA. Radioassays for the proteolytic enzyme secreted by living eggs of *Schistosoma mansoni*. *Int J Parasitol*, **1974**. 4:681–683.
173. Smyth D. *The Physiology of Trematodes, first ed.* Oliver and Boyd, Edinburgh and London, **1966**.
174. Smyth D and Halton D. *The Physiology of Trematodes, second ed.* Cambridge University Press, Cambridge, **1983**.
175. Smyth JD and Clegg JA. Egg-shell formation in trematodes and cestodes. *Exp Parasitol*, **1959**. 8:286–323.
176. Stadecker MJ and Hernandez HJ. The immune response and immunopathology in infection with *Schistosoma mansoni*: a key role of major egg antigen sm-p40. *Parasite Immunol*, **1998**. 20:217–221.
177. Stein PD and Sabbah HN. Measured turbulence and its effect on thrombus formation. *Circ Res*, **1974**. 35:608–614.
178. Steinfelder S, Andersen JF, Cannons JL, Feng CG, Joshi M, Dwyer D, Caspar P, Schwartzberg PL, Sher A and Jankovic D. The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). *J Exp Med*, **2009**. 206:1681–1690.
179. Taylor DD and Gercel-Taylor C. Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. *Semin Immunopathol*, **2011**. 33:441–454.
180. ten Hove RJ, Verweij JJ, Vereecken K, Polman K, Dieye L and van Lieshout L. Multiplex real-time PCR for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* infection in stool samples collected in northern Senegal. *Trans R Soc Trop Med Hyg*, **2008**. 102:179–185.
181. Tibbling G, Link H and Öhman S. Principles of albumin and IgG analyses in neurological disorders. I. Establishment of reference values. *Scand J Clin Lab Invest*, **1977**. 37:385–390.
182. Tielens AGM, van de Pas FA, van den Heuvel JM and van den Bergh SG. The aerobic energy metabolism of *Schistosoma mansoni* miracidia. *Mol Biochem Parasitol*, **1991**. 46:181–184.
183. Vale TC, de Sousa-Pereira SR, Ribas JGR and Lambertucci JR. Neuroschistosomiasis mansoni: Literature review and guidelines. *Neurologist*, **2012**. 18:333–342.
184. van Balkom BWM, van Gestel RA, Brouwers JFHM, Krijgsveld J, Tielens AGM, Heck AJR and van Hellemond JJ. Mass spectrometric analysis of the *Schistosoma mansoni* tegumental sub-proteome. *J Proteome Res*, **2005**. 4:958–966.
185. van de Vijver KK, Deelder AM, Jacobs W, Marck EAV and Hokke CH. LacdiNAc- and LacNAc-containing glycans induce granulomas in an *in vivo* model for schistosome egg-induced hepatic granuloma formation. *Glycobiology*, **2006**. 16:237–243.
186. van de Vijver KK, Hokke CH, van Remoortere A, Jacobs W, Deelder AM and Marck EAV. Glycans of *Schistosoma mansoni* and keyhole limpet haemocyanin induce hepatic granulomas *in vivo*. *Int J Parasitol*, **2004**. 34:951–961.

187. van der Kleij D, Latz E, Brouwers JFHM, Kruize YCM, Schmitz M, Kurt-Jones EA, Espevik T, de Jong EC, Kapsenberg ML, Golenbock DT, Tielens AGM and Yazdanbakhsh M. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J Biol Chem*, **2002**. 277:48122–48129.
188. van Die I, van Liempt E, Bank CMC and Schiphorst WECM. Interaction of schistosome glycans with the host immune system. *Adv Exp Med Biol*, **2005**. 564:9–19.
189. van Gool T, Vetter H, Vervoort T, Doenhoff MJ, Wetsteyn J and Overbosch D. Serodiagnosis of imported schistosomiasis by a combination of a commercial indirect hemagglutination test with *Schistosoma mansoni* adult worm antigens and an enzyme-linked immunosorbent assay with *S. mansoni* egg antigens. *J Clin Microbiol*, **2002**. 40:3432–3437.
190. van Hellemond JJ, Retra K, Brouwers JFHM, van Balkom BWM, Yazdanbakhsh M, Shoemaker CB and Tielens AGM. Functions of the tegument of schistosomes: clues from the proteome and lipidome. *Int J Parasitol*, **2006**. 36:691–699.
191. van Remoortere A, Bank CMC, Nyame AK, Cummings RD, Deelder AM and van Die I. *Schistosoma mansoni*-infected mice produce antibodies that cross-react with plant, insect, and mammalian glycoproteins and recognize the truncated biantennary *N*-glycan Man3GlcNAc2-R. *Glycobiology*, **2003**. 13:217–225.
192. van Remoortere A, Hokke CH, van Dam GJ, van Die I, Deelder AM and van den Eijnden DH. Various stages of *Schistosoma* express Lewis(x), LacdiNAc, GalNAcβ1-4 (Fucα1-3)GlcNAc and GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc carbohydrate epitopes: detection with monoclonal antibodies that are characterized by enzymatically synthesized neoglycoproteins. *Glycobiology*, **2000**. 10:601–609.
193. van Riet E, Hartgers FC and Yazdanbakhsh M. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology*, **2007**. 212:475–490.
194. Waite JH. Precursors of quinone tanning: dopa-containing proteins. *Methods Enzymol*, **1995**. 258:1–20.
195. Wells KE and Cordingley JS. *Schistosoma mansoni*: eggshell formation is regulated by pH and calcium. *Exp Parasitol*, **1991**. 73:295–310.
196. Wharton DA. The production and functional morphology of helminth egg-shells. *Parasitology*, **1983**. 86:85–97.
197. WHO Expert Committee. Prevention and control of schistosomiasis and soil-transmitted helminthiasis. *World Health Organ Tech Rep Ser*, **2002**. 912:i-vi, 1–57.
198. Wichmann D, Panning M, Quack T, Kramme S, Burchard GD, Grevelding C and Drosten C. Diagnosing schistosomiasis by detection of cell-free parasite DNA in human plasma. *PLoS Negl Trop Dis*, **2009**. 3:e422.
199. Wiest PM, Tartakoff AM, Aikawa M and Mahmoud AA. Inhibition of surface membrane maturation in schistosomula of *Schistosoma mansoni*. *Proc Natl Acad Sci U S A*, **1988**. 85:3825–3829.
200. Williams DL, Asahi H, Botkin DJ and Stadecker MJ. Schistosome infection stimulates host CD4(+) T helper cell and B-cell responses against a novel egg antigen, thioredoxin peroxidase. *Infect Immun*, **2001**. 69:1134–1141.

201. Wilson MS, Mentink-Kane MM, Pesce JT, Ramalingam TR, Thompson R and Wynn TA. Immunopathology of schistosomiasis. *Immunol Cell Biol*, **2007**. 85:148–154.
202. Wilson RA. Cercariae to liver worms: development and migration in the mammalian host. In: Rollinson D and Simpson AJG, eds., *The biology of schistosomes, from genes to latrines*, 115–146. Academic Press, London, **1987**.
203. Wu YP, Lenting PJ, Tielens AGM, de Groot PG and van Hellemond JJ. Differential platelet adhesion to distinct life-cycle stages of the parasitic helminth *Schistosoma mansoni*. *J Thromb Haemost*, **2007**. 5:2146–2148.
204. Xu J, Liu AP, Guo JJ, Wang B, Qiu SJ, Sun H, Guan W, Zhu XQ, Xia CM and Wu ZD. The sources and metabolic dynamics of *Schistosoma japonicum* DNA in serum of the host. *Parasitol Res*, **2013**. 112:129–133.
205. Xu X and Caulfield JP. Characterization of human low density lipoprotein binding proteins on the surface of schistosomula of *Schistosoma mansoni*. *Eur J Cell Biol*, **1992**. 57:229–235.
206. Yang J, Qiu C, Xia Y, Yao L, Fu Z, Yuan C, Feng X and Lin J. Molecular cloning and functional characterization of *Schistosoma japonicum* enolase which is highly expressed at the schistosomulum stage. *Parasitol Res*, **2010**. 107:667–677.
207. Yong MK, Beckett CL, Leder K, Biggs BA, Torresi J and O'Brien DP. Long-term follow-up of schistosomiasis serology post-treatment in Australian travelers and immigrants. *J Travel Med*, **2010**. 17:89–93.
208. Zauner G, Hoffmann M, Rapp E, Koeleman CAM, Dragan I, Deelder AM, Wuhrer M and Hensbergen PJ. Glycoproteomic analysis of human fibrinogen reveals novel regions of O-glycosylation. *J Proteome Res*, **2012**. 11:5804–5814.

Samenvatting

Schistosomen zijn parasitaire wormen die leven in bloedvaten van zoogdieren. Wereldwijd zijn meer dan 200 miljoen mensen geïnfecteerd met een van de 5 schistosomensoorten die infectieus zijn voor de mens. Bovendien zijn er nog vele soorten die dieren (waaronder vee) infecteren. Naast medische problemen leiden infecties bij mens en dier ook tot sociale en economische schade.

Dit proefschrift gaat over *Schistosoma mansoni*. Besmetting van de mens vindt plaats bij zoetwatercontact. *Schistosoma* larven (cercariae) die in het water zwemmen dringen de huid binnen. Via de bloedbaan vinden de parasieten hun weg naar de lever, waar de inmiddels volwassen geworden wormen paren en van waaruit zij naar de bloedvaten rond de darm migreren. In de bloedvaten leven mannelijke en vrouwelijke wormen gepaard samen. Zij kunnen daar meerdere jaren en soms decennia lang overleven. Intussen legt het vrouwtje zo'n 300 eieren per dag. Een deel van de eieren passeert de bloedvatwand en de darmwand en wordt met de ontlasting uitgescheiden. De overige eieren worden door de bloedstroom meegevoerd en komen uiteindelijk in de lever vast te zitten. Wanneer uitgescheiden eieren in het water belanden en uitkomen, kunnen de larven (miracidia) de tussengastheer, een zoetwaterslak, infecteren. In de slakken vermenigvuldigen de larven zich en ontwikkelen ze zich tot voor de mens infectieuze cercariae. De zoetwaterslakken die gevoelig zijn voor *Schistosoma* infecties komen alleen voor in tropische wateren. Vandaar dat schistosomiasis, de naam van de ziekte veroorzaakt door *Schistosoma* wormen, een tropische ziekte is.

Schistosoma eieren zijn erg immunogeen, hetgeen betekent dat ze een sterke afweerreactie uitlokken. Deze afweerreactie kan ernstige schade aan de omliggende weefsels veroorzaken. De eischalen, de buitenkant van de eieren, zijn continu in interactie met de componenten van het afweersysteem in de bloedstroom en in de weefsels. Daarom is het van belang de samenstelling van deze eischaal goed in kaart te brengen. De eischaal bestaat uit gecrosslinkte eiwitten. Deze vormen een ijzersterke constructie die door geen enkele protease kan worden afgebroken. De crosslinking die hiervoor zorgt heet "quinone tanning" en vindt plaats bij tyrosine-

residuen in eiwitten. Onder de eischaal bevinden zich nog enkele cellulaire en non-cellulaire lagen. Midden in het ei zit het miracidium.

In **hoofdstuk 2** staan de resultaten beschreven van de eiwit-analyse van de eischalen. Er moest een zuiver product verkregen worden alvorens de eischalen geanalyseerd konden worden. De eischalen zijn in 4 stappen van oplopende agressiviteit behandeld om ze vrij te maken van aanhangende eiwitten afkomstig van andere ei-componenten of van de gastheer. Het resulterende materiaal bestond uitsluitend uit gecrosslinkte eiwitten en kreeg de naam gezuiverde eischaalfragmenten.

Na elke zuiveringsstap zijn eischalen geanalyseerd met de massaspectrometer (MS). Het aantal geïdentificeerde eiwitten loopt af van 118 eiwitten direct na ei-isolatie uit levers tot 45 eiwitten in de gezuiverde eischaalfragmenten. De lijst van 45 eiwitten bestaat grotendeels uit niet-structurele eiwitten, waaronder een aantal bekende *Schistosoma* antigenen.

In de jaren '80 en '90 zijn vrouwspecifieke eiwitten geïdentificeerd. Zij kregen de naam "putative eggshell proteins" p14 en p48. Deze eiwitten zijn echter niet teruggevonden bij de MS-analyse van de gezuiverde eischaalfragmenten. Ze bevatten veel tyrosines en zijn daardoor waarschijnlijk sterk gecrosslinkt en als gevolg daarvan onherkenbaar veranderd. Opvallend is het hoge percentage glycine in p14: 44%. Aminozuur-analyse van de gezuiverde eischaalfragmenten toonde aan dat deze voor 36% uit glycine bestaan. Dit is een sterke aanwijzing dat p14 inderdaad in de eischalen zit en daarmee ook de grootste component van de eischaal vormt. Door middel van antilichamen tegen p14 is dit eiwit daadwerkelijk aangetoond in de gezuiverde eischaalfragmenten.

Al met al tonen deze resultaten aan dat eischalen weliswaar grotendeels bestaan uit eischaal-specifieke eiwitten, maar ook allerlei andere eiwitten bevatten. Waarschijnlijk waren die andere eiwitten min of meer toevallig ter plekke aanwezig ten tijde van eischaalformatie.

Hoofdstuk 3 beschrijft de structuur van de eischalen. Wanneer een ei door een vrouwtje wordt uitgescheiden, bestaat dit slechts uit een eischaal, een embryo en een klompje vitellinecellen. Gedurende tenminste een week ontwikkelen zich het miracidium en de cellulaire en acellulaire lagen die het miracidium omringen. Een deel van de eiwitten die in hoofdstuk 2 zijn geïdentificeerd als onderdeel van de eischaal zijn bekende *Schistosoma* antigenen. In dit hoofdstuk wordt aangetoond dat eischalen zelf immunogeen zijn en zowel *in vitro* als *in vivo* door antilichamen herkend worden. De aanwezigheid van niet-afbreekbare antigen-antilichaam

complexen leidt tot chronische ontstekingen en zorgt voor het ontstaan van granulomen.

Verder is bekend dat het afweersysteem bijdraagt aan de uitscheiding van eieren in de ontlasting. Het is waarschijnlijk dat naast de door het ei uitgescheiden eiwitten, de eischaal zelf hierin een rol speelt door antilichamen te binden en T-cellen te activeren.

Het is bekend dat naast antilichamen en afweercellen ook bloedplaatjes aan eischalen binden. In **hoofdstuk 4** wordt aangetoond dat von Willebrand factor (VWF), een belangrijke factor voor stolling en een activator van bloedplaatjes, rechtstreeks bindt aan eischalen. Ook fibronectine en fibrinogeen, andere factoren die betrokken zijn bij de stolling, blijken aan eischalen te binden. Stolling is een proces dat snel wordt bewerkstelligd. De stollingsfactoren die aan eischalen binden kunnen een brug vormen tussen de eischaal en de bloedvatwand. Zonder een dergelijke, snel aangelegde, verbinding zou het ei nadat het door het vrouwtje gelegd is snel door het bloedvat stromen en geen gelegenheid hebben door de wand van het bloedvat te dringen en zo de bloedsomloop te verlaten.

Wanneer eieren op een afwijkende plaats in het lichaam terecht komen, spreken we van ectopische schistosomiasis. Dit kan tot klachten leiden door de afweerreactie die rond de eieren ontstaat waardoor omliggende structuren samengedrukt of beschadigd worden. Een van de ernstigste vormen van ectopische schistosomiasis is neuroschistosomiasis, waar de aanwezigheid van eieren in het centrale zenuwstelsel binnen enkele weken kan leiden tot ernstige, soms onomkeerbare neurologische schade. Snelle diagnostiek is daarom van groot belang. Naast neuroschistosomiasis zijn er nog tal van mogelijke (infectieuze) oorzaken van neurologische uitval. Omdat de behandeling van neuroschistosomiasis onder andere bestaat uit een hoge dosis corticosteroïden, die een afweeronderdrukkende werking hebben, is het belangrijk neuroschistosomiasis met zekerheid aan te tonen of eventuele andere infectieuze oorzaken van de neurologische uitval uit te sluiten. De gouden standaard voor diagnose van neuroschistosomiasis is het aantonen van eieren in het centrale zenuwstelsel. Dit is een risicovolle procedure en wordt daarom zelden toegepast.

Bij schade aan de bloed-hersenbarrière zullen eiwitten vanuit het bloed in de liquor (het hersenvocht) lekken. De liquor zal dan meer eiwitten bevatten dan gewoonlijk. Als de neurologische aandoening veroorzaakt wordt door een infectieuze verwekker in het centrale zenuwstelsel, dan zullen bovendien binnen in het centrale zenuwstelsel (intrathecaal) antilichamen worden aangemaakt tegen

de betreffende verwekker. Wanneer alle plasma-eiwitten in ongeveer dezelfde verhoudingen aanwezig zijn in de liquor als in het plasma, is intrathecale antilichaamproductie niet waarschijnlijk. Maar als in de liquor antilichamen sterker vertegenwoordigd zijn dan andere plasma-eiwitten wijst dit op een infectieuze oorzaak van de neurologische aandoening. Bij neuroschistosomiasis zijn de eieren de veroorzakers van de schade en niet de wormen. In dat geval is het te verwachten dat er relatief meer anti-ei-antilichamen dan anti-worm-antilichamen in de liquor. Een index waarbij deze ei-worm antilichamenratio genormaliseerd werd met de verhouding anti-ei/anti-worm antilichamen in het serum, leverde sterke aanwijzingen voor intrathecale antilichaamproductie tegen *Schistosoma* eieren in patiënten met neuroschistosomiasis. Dit staat beschreven in **hoofdstuk 5** van dit proefschrift.

Niet alleen de eieren bevinden zich in constante interactie met de gastheer. De volwassen wormen die in de bloedvaten leven worden ook continu blootgesteld aan het afweersysteem. Toch weten de wormen een ernstige afweerreactie te onderdrukken of te vermijden. De buitenkant van de wormen, het tegument, bestaat uit een laagje gefuseerde cellen bedekt door een dubbele bilaag van fosfolipiden. In **hoofdstuk 6** wordt een analyse gegeven van de fosfolipidensamenstelling van geïsoleerd tegument. Deze wordt vergeleken met de fosfolipidensamenstelling van de gehele wormen en die van de membranen van bloedcellen van de gastheer. De fosfolipidensamenstelling van schistosomen blijkt sterk af te wijken van die van membranen van de gastheercellen. Daarbij valt op dat het tegument extra verrijkt is aan bepaalde parasiet-specifieke fosfolipiden ten opzichte van de gehele worm. Hieronder vallen fosfolipiden met langere vetzuurstaarten en met meer dubbele bindingen. Daarnaast zijn ook de lysofosfolipiden sterk verrijkt in het tegument. Dit geldt met name voor lysofosfatidylserine (lysoPS) en lysofosfatidylethanolamine (lysoPE) met een vetzuurstaart van eicosaanzuur (20:1). Deze lysofosfolipiden werden niet meetbaar uitgescheiden in *in vitro* incubaties en zijn ook niet gevonden in bloed plasma van *Schistosoma*-geïnfecteerde hamsters.

Zowel *Schistosoma* eieren als *Schistosoma* wormen zijn continu in interactie met de gastheer. De eischaal en het tegument zijn hele specifieke structuren wat betreft bouw en samenstelling. Verdere analyse van deze structuren kan waardevolle informatie verschaffen over de biologie van schistosomen. Bovendien zijn deze structuren, vanwege hun bijzondere samenstellingen, voor de hand liggende aangrijpingspunten voor de ontwikkeling van vaccins of nieuwe anti-*Schistosoma* therapieën.

Appendices

Acknowledgments

Curriculum Vitae

List of publications

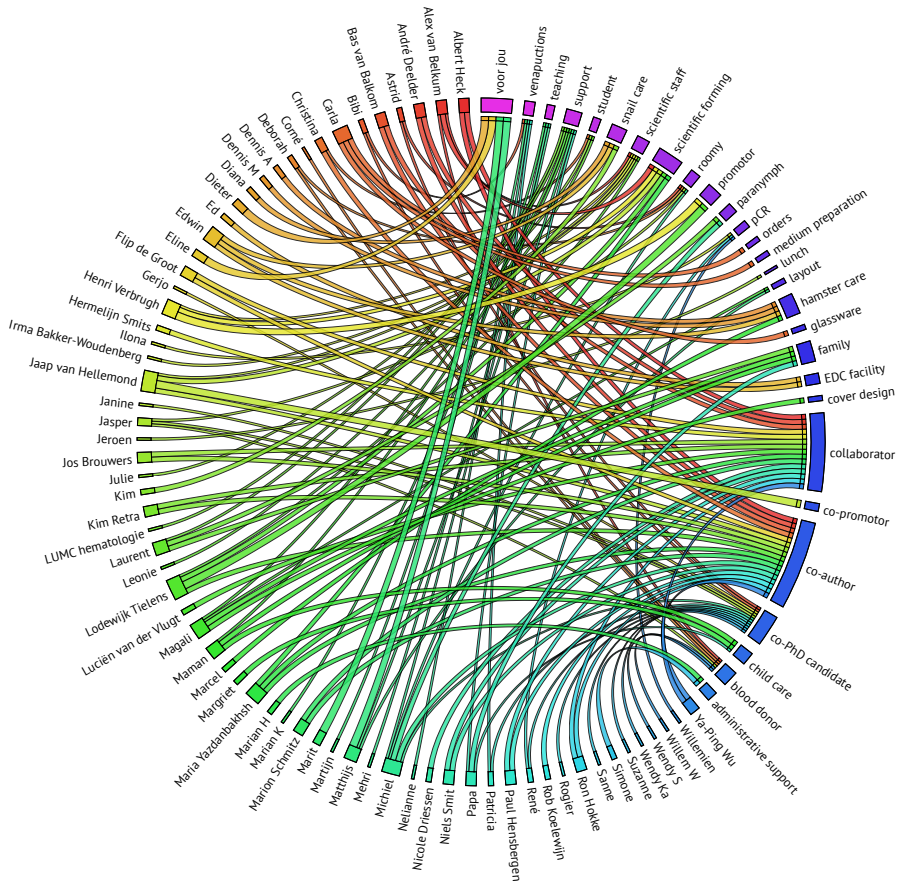
Portfolio

Appendix A

Acknowledgements

A larger image of this figure is available on
<http://saskia.mired.nl/thesis/acknowledgments.html>





Hoewel mijn naam op de voorkant van dit proefschrift staat, is het mede tot stand gekomen door bijdragen van velen. Dank aan allen.

Appendix B

Curriculum vitae

Saskia Marie-Claude Annick de Walick was born in 1981 in Voorburg, the Netherlands. After secondary school, she started her study medicine at the Leiden University. As an undergraduate student she did research at the Department of Parasitology of the Leiden University Medical Center (LUMC) under supervision of dr. L. van Lieshout and dr. J.J. Verweij. This project comprised epidemiology of diagnosed malaria cases in the LUMC as well as laboratory work on the development of a real-time PCR assay for the diagnosis of malaria and differentiation of *Plasmodium* spp. Still during her studies, she did an epidemiologic study on the relation between body height reduction as a measure of osteoporosis and bone fracture occurrence in elderly people. This study was performed on the Leiden 85-plus Study data of prof.dr. R.G.J. Westendorp under his supervision (LUMC). For her master's thesis she went to the Immunology and Infection laboratory of dr. C.R. Engwerda at the Queensland Institute of Medical Research in Brisbane, Australia. Here, she investigated the role of dendritic cells in the development of experimental cerebral malaria. After acquiring her master's degree she started her PhD in 2008 at the Department of Medical Microbiology and Infectious Diseases of the Erasmus Medical Center in Rotterdam under supervision of prof.dr. A.G.M. Tielens and dr. J.J. van Hellemond. The results of her work on *Schistosoma mansoni* and the interactions with the host are presented in this thesis. She currently works on clinical trials at the Department of Hematology of the LUMC.

Appendix C

List of publications

deWalick S, Amante FH, McSweeney KA, Randall LM, Stanley AC, Haque A, Kuns RD, MacDonald KP, Hill GR, Engwerda CR. Cutting edge: conventional dendritic cells are the critical APC required for the induction of experimental cerebral malaria. *J Immunol*, **2007**. May 15;178(10):6033–7.

deWalick S, Bexkens ML, van Balkom BW, Wu YP, Smit CH, Hokke CH, de Groot PG, Heck AJ, Tielens AGM, van Hellemond JJ. The proteome of the insoluble *Schistosoma mansoni* eggshell skeleton. *Int J Parasitol*, **2011**. Apr;41(5):523–32.

deWalick S, Tielens AGM, van Hellemond JJ. *Schistosoma mansoni*: the egg, biosynthesis of the shell and interaction with the host. *Exp Parasitol*, **2012**. Sep;132(1):7–13.

deWalick S, Hensbergen PJ, Bexkens ML, Grosserichter-Wagener C, Hokke CH, Deelder AM, de Groot PG, Tielens AGM, van Hellemond JJ. Binding of von Willebrand factor and plasma proteins to the eggshell of *Schistosoma mansoni*. *Int J Parasitol*, **2014**. Apr;44(5):263–8.

Retra K, **deWalick S**, Schmitz M, Yazdanbakhsh M, Tielens AGM, Brouwers JFHM, van Hellemond JJ. The tegumental surface membranes of *Schistosoma mansoni* are enriched in parasite-specific phospholipid species. *Int J Parasitol*, **2015**. Accepted for publication.

Hempel HL, van Hellemond JJ, Meeuwis CA, Biermann K, **deWalick S**, ten Hove I. Human dirofilariasis due to *Dirofilaria repens* in the parotid gland. A Dutch case report. *Submitted for publication*.

Appendix D

Portfolio

PhD portfolio drs. S.M.A. de Walick

PhD period:	2008-2012
ErasmusMC Department:	Medical Microbiology and Infectious Diseases
Research school:	Erasmus Postgraduate School Molecular Medicine
Promotor:	Prof.dr. A.G.M. Tielens and Prof.dr. H.A. Verbrugh
Copromotor:	Dr. J.J. van Hellemond

1. PhD training

General courses

Biomedical research techniques VII	2008
Biomedical English Writing and Communication	2009

Specific courses

The 2nd symposium and workshops on molecular microbiology of infectious diseases	2008
Basic course on R	2010
Workshop on Adobe Photoshop and Illustrator CS4	2010

Seminars and workshops

Department journal club	2008-2012
Department seminars	2008-2012

(Inter)national conferences

Molecular and Cellular Biology of Helminth Parasites V, Hydra, Greece (poster presentation)	2008
Spring Meeting of the Dutch Society for Medical Microbiology, Papendal, The Netherlands (oral presentation)	2009
Spring Meeting of the Netherlands Society for Parasitology, Kerkrade, The Netherlands (oral presentation)	2009
11th International symposium on flatworm biology, Hasselt, Belgium (oral presentation)	2010
British Society of Parasitology spring Meeting, Cardiff, Wales, UK (oral presentation)	2010
Molecular and Cellular Biology of Helminth Parasites VI, Hydra, Greece (poster presentation)	2010
British Society of Parasitology spring Meeting, Nottingham, UK (oral presentation)	2011

2. Teaching

Supervising bachelor's and master's theses	2009-2012
Teaching assistant in course for medical students "VO infectieziekten"	2009-2012

