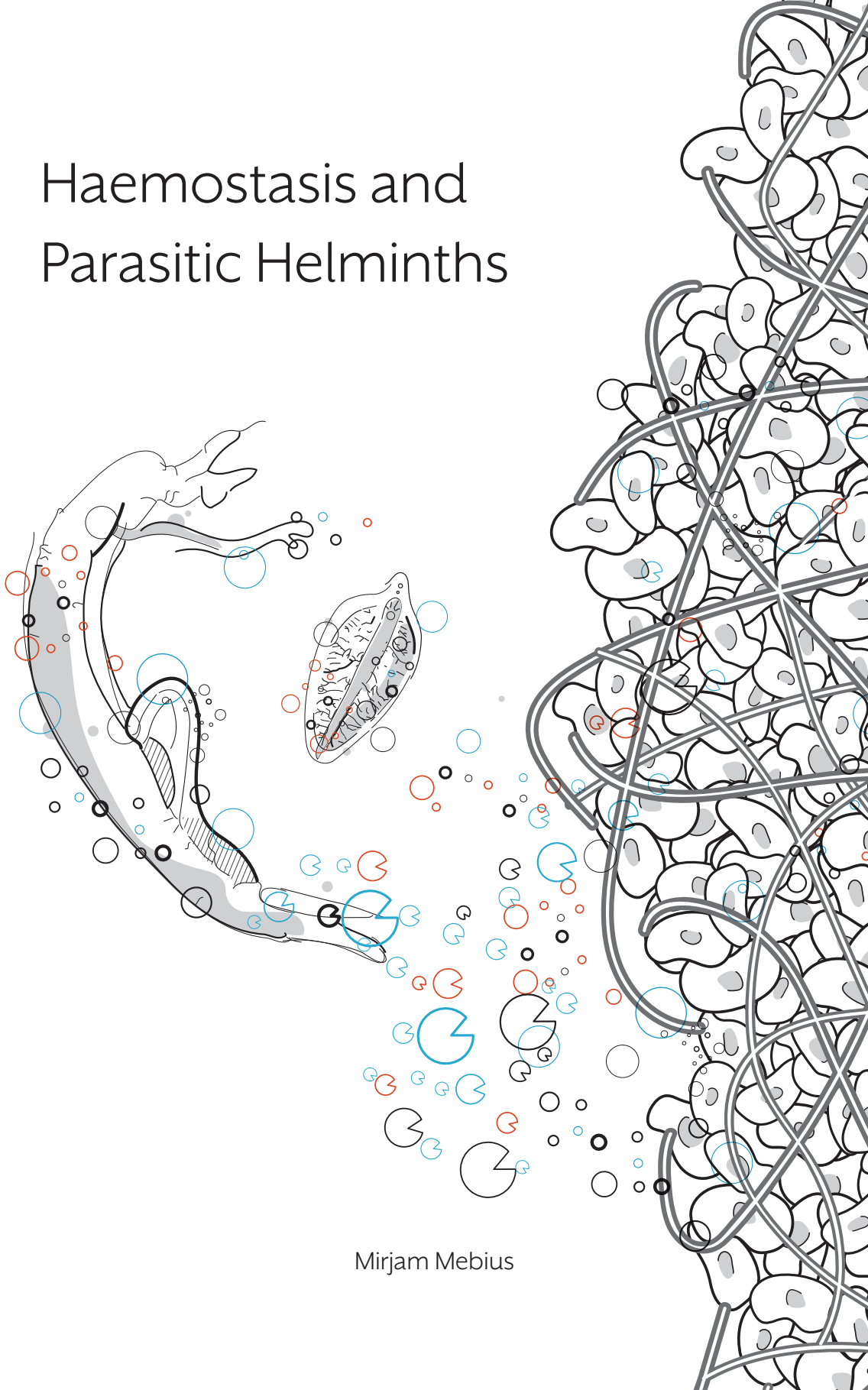
Haemostasis and Parasitic Helminths



Mirjam Mebius

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Haemostasis and Parasitic Helminths

Haemostase en parasitaire helminten

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“No matter - try again - fail again - fail better”

Job Koelewijn, 2001, ‘Formula B’

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CHAPTER 1

General introduction

Introduction to parasites in human disease

In biology, the term "parasitism" describes a relationship between two organisms in which one organism (the parasite) lives at the expense of the other organism (the host). The parasite benefits from the relationship through easy access to nutrients and a very constant environment. The loss of nutrients to the parasite and damage to the host, either mechanical or through stimulation of a damaging immune response that results from this relationship, are disadvantageous for the host.

Parasitism is a highly common way of living: it is estimated that between 30% and 71% of all named species are parasitic [1]. Although many viruses, bacteria, and fungi have a parasitic lifestyle, the term "parasite" is limited to protozoa and multicellular eukaryotic organisms with a parasitic lifestyle. Humans alone can be infected with hundreds of different parasite species and evidence of parasitic infections in humans has been dated back to prehistoric times [2]. Parasites can be divided into three main categories: 1) unicellular parasitic protozoa, such as malaria, 2) multicellular parasitic worms (helminths), such as schistosomes and *Fasciola*, and 3) ectoparasites, such as ticks and mosquitos. This thesis will only focus on helminths, and more specifically on schistosomes and *Fasciola* parasites, and therefore protozoa and ectoparasites will not be discussed. Schistosomiasis, caused by parasitic helminths called schistosomes, is the world's second most important parasitic disease of public health importance after malaria [3]. Schistosomes are responsible for over 200 million infections worldwide and an estimated 280,000 deaths yearly in sub-Saharan Africa alone [4]. Additionally, parasitic infections of cattle cause major economic losses and zoonotic parasitic infections affect human health [5]. In tropical regions, fascioliasis, caused by parasitic helminths called *Fasciola*, is considered the most important helminth infection in cattle, affecting 30-90% in Africa, 25-100% in India, and 25-90% in Indonesia [5,6]. Not only cattle is affected by this parasite: the WHO estimates that worldwide at least 2.4 million humans suffer from fascioliasis, with several million people at risk in over 70 countries [5]. However, this may be an underestimation as the global burden of fascioliasis is approximated to be between 35 and 72 million people [7]. It is thus clear that parasitic infections form a serious health and economic burden worldwide.

Schistosomiasis

The tropical parasitic disease schistosomiasis, or bilharzia, is caused by blood-dwelling parasitic trematodes (flatworms) of the genus *Schistosoma*. Although five schistosome species can cause infection in human, the major disease-causing species are *Schistosoma mansoni, Schistosoma haematobium* and *Schistosoma japonicum* [8,9].

Morphology of the adult schistosome

Schistosomes have separate sexes and the adult worms live in pairs in blood vessels of their host. The male worm will fold its body into a groove in which it embraces the slightly longer and thinner female, which gives the worms their cylindrical appearance. Adult schistosomes vary in length from 7 to 20 mm and have two terminal suckers required for attachment to the blood vessel wall. The outer surface of the worm is formed by a complex tegument layer, which is a 1 to 3 μm thick syncytial layer covered by a double lipid bilayer (the membranocalyx). This tegument layer is thought to play an important role in evasion of host defence systems by the parasite [10]. Schistosomes have a blind digestive tract in which digestion of blood components takes place, after which degraded products are expelled into the host bloodstream by regurgitation [11].

The schistosomal life cycle

Human *Schistosoma* species have a complex life cycle (Figure 1) which involves two different hosts: a tropical freshwater snail as the intermediate host and humans as the final host. Adult worms live in the mesenteric (*S. mansoni* and *S. japonicum* species) or perivesicular veins (*S. haematobium* species) of their human host, where they mate and produce hundreds to thousands of larva-containing eggs per day [8]. About half of the eggs get trapped in the tissues of the liver (*S. mansoni* and *S. japonicum* species) or wall of the urine bladder (*S. haematobium* species), where they elicit a strong immune response leading to granuloma formation and tissue damage [8]. The other eggs migrate through the vessel wall and lining of the intestine (*S. mansoni* and *S. japonicum* species) or urinary bladder (*S. haematobium* species), after which they are excreted with faeces or urine. Water contact triggers the release of the ciliated larva, called miracidium, from the egg. The miracidium infects fresh-water snails that function as the intermediate host. Each *Schistosoma* species infects a specific snail species; *S. mansoni* infects *Biomphalaria* snails, *S. japonicum* infects *Oncomelania* snails, and *S. haematobium* infects *Bulinus* snails [9]. Inside the snail, the miracidia reproduce asexually to produce sporocysts that mature into cercarial larvae. After 4-6 weeks, the cercariae leave the snail in search for their definitive host, humans. Humans are infected after contact with infected water, upon which the cercariae will penetrate the skin. Skin penetration results in the loss of the cercarial tail and transforms the larvae into migrating juvenile worms, the schistosomula. These schistosomula migrate through the bloodstream to the lungs and then to the portal vein, where they mature into adult schistosomes. The adult schistosomes mate and migrate to the perivesicular or mesenteric veins, where they live on average 3 to 5 years, but their lifespan can be as long as 30 years [8].

Symptoms of disease

Three distinct disease stages can be distinguished during the course of an infection with *Schistosoma* species: swimmer's itch, acute schistosomiasis and chronic schistosomiasis [8]. Although, schistosome infections often occur without notice, symptoms may be present at all stages of the disease [8,9].

Penetration of the skin by cercariae can trigger a local skin reaction which is called swimmer's itch [12]. Symptoms arise shortly after contact with infected water and may persist for several hours up to a couple of days [13]. Also non-human schistosome species, in particularly bird schistosome species, can cause swimmer's itch. Due to the similarities in symptoms, it is often difficult to distinguish whether the skin reactions were caused by bird or human schistosome species [14].

Acute schistosomiasis, also called Katayama fever, can occur 2-10 weeks after a primary infection [15]. Symptoms are flu-like and include fever, fatigue, muscle pain, and non-productive cough. Acute schistosomiasis results from a T-helper-1 cell (Th1) mediated hypersensitivity response towards the migrating and maturing schistosomula. High eosinophilia and patchy infiltrates on chest radiography are characteristic of this stage.

In chronic schistosomiasis, symptoms are not caused by the adult parasite, but by entrapment of eggs in the lining of the intestine or bladder during egg-excretion, or entrapment of eggs that fail to extravasate in organs such as the liver, spleen, lungs or central nervous system. The entrapped eggs elicit a strong immune response leading to granuloma formation and fibrosis [8]. The severity of symptoms is therefore determined by both the intensity and duration of the infection as well as host factors that determine individual immune responses.

Several forms of chronic schistosomiasis can be distinguished, based on the *Schistosoma* species that causes the infection. Infections with *S. haematobium*, which reside in the perivesicular veins, causes urinary schistosomiasis. Urinary schistosomiasis is, in early stages, characterized by the presence of blood in the urine (haematuria). Later symptoms include calcification of the bladder, urodynamic disorders, and renal dysfunction. Additionally, there is a strong association between urinary schistosomiasis and the development of bladder cancer [9]. Intestinal, hepatic, and hepatosplenic schistosomiasis are mostly caused by *S. mansoni* and *S. japonicum* infections, although hepatic and hepatosplenic disease can also occur in severe cases of schistosomiasis haematobium [16]. Intestinal schistosomiasis is characterized by abdominal pain and diarrhoea with or without bloody stools. Hepatic schistosomiasis or early inflammatory schistosomiasis is caused by an early reaction to entrapped eggs in the liver, leading to mild or diffuse fibrosis and enlargement of the liver. Hepatosplenic schistosomiasis or fibrotic hepatic schistosomiasis develops after years of chronic infection. It results from periportal collagen deposition, leading to periportal fibrosis. Periportal fibrosis leads

to the development of portal hypertension, splenomegaly, and gastro-oesophagal varices, which can result in fatal bleeding [8].

Ectopic schistosomiasis can develop in tissues that are accidentally reached by adult parasites or eggs. Ectopic schistosomiasis includes pulmonary, genital and neuroschistosomiasis, and symptoms are caused by inflammatory responses and fibrosis in response to the parasite or eggs [8].

Diagnosis

The gold standard for the diagnosis of active schistosomiasis is the detection of viable eggs in urine (*S. haematobium*) or faeces (*S. mansoni* and *S. japonicum*). As direct microscopic detection of eggs is not very sensitive, sample concentrations methods are usually required, although even then light infections can still be missed [8].

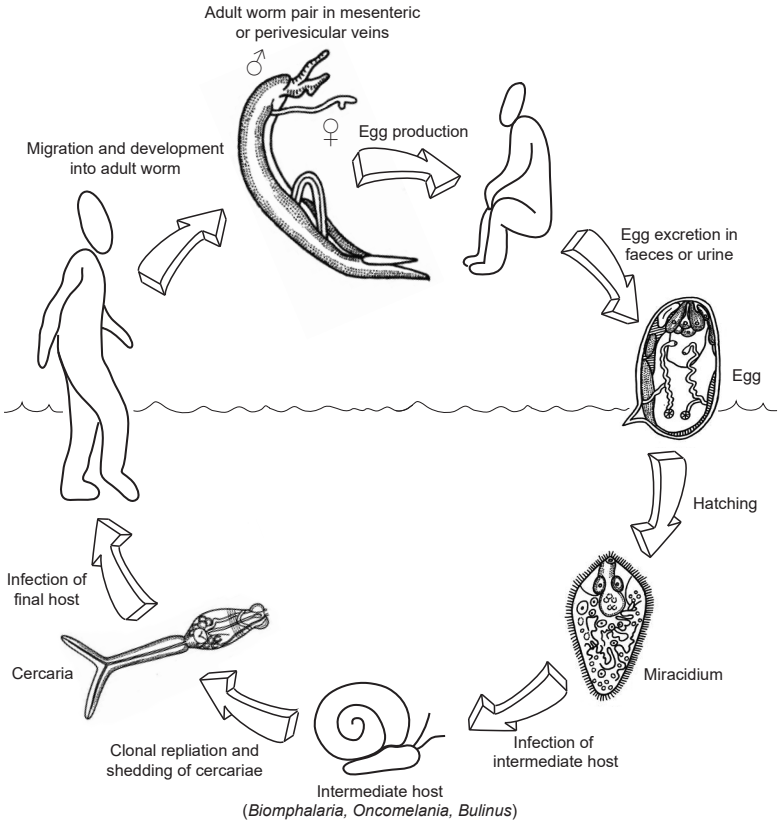

Figure 1. The life cycle of *Schistosoma* species.

Seroconversion usually happens between 4 to 8 weeks after infection, so at this stage serological tests could confirm schistosomiasis. Serological tests are highly sensitive in detecting antibodies against soluble worm antigen or, later in infection, crude soluble egg antigen with ELISA, indirect haemagglutination, or immunofluorescence [8]. However, a major drawback of serological tests is that antibodies may be present long after the infections has been cleared, at least for two years, making discrimination between a past or ongoing infections difficult.

Alternatively, detection of circulating schistosomal antigens, in particular circulating cathodic antigen (CCA) and circulating anodic antigen (CAA), can be performed in urine using lateral-flow (dipstick) tests [17]. As these circulating antigens are only produced by viable schistosomes, detection of these antigens can be used to detect active schistosome infections. Recent improvements in sensitivity and sample concentration methods have led to recommendations by the WHO for the use of these dipstick tests in mapping and monitoring programmes [9,17–19].

Other diagnostic tools include detection of parasitic DNA by real-time PCR, and visualization of tissue pathology through endoscopy, (contrast)radiology, CT, and MRI. However, due to high costs and technical issues these techniques are rarely performed routinely in low-income endemic areas [8,9].

Treatment and control

Praziquantel is the drug of choice for treatment of schistosomiasis, as it is low-cost, safe and effective in a single oral dose against all schistosome species [8]. While praziquantel is effective against adult worms, it has little or no effect on eggs or juvenile worms [9]. Although the antihelminthic activity of praziquantel has been discovered in 1972, its working mechanism is still not fully understood [20]. It is known that the drug initiates a rapid influx of Ca^{2+} and causes paralysis in the mature parasites. This is accompanied by blebbing of the tegumental and subtegumental structures, resulting in disruption of the tegument and exposure of parasite surface antigens. This is thought to lead to recognition and clearance of the parasite by the host immune system, and would explain why an effective host antibody response is required for full efficacy of praziquantel [9,20]. Praziquantel does not prevent reinfection, therefore, in endemic areas where reinfection is likely, treatment is given at regular intervals in mass drug administration programs. In travellers or patients suffering from Katayama fever, two doses of praziquantel are administered with an interval of 4 to 6 weeks to eradicate all parasites, including maturing juveniles. Additionally, corticosteroids can be used to dampen immune responses in Katayama fever and cases of neuroschistosomiasis [8].

Control and elimination of schistosomiasis could be achieved by implementing (preventative) mass drug administration programmes, combined with interruption of transmission by behavioural changes in hygiene habits, providing access to safe

water, sanitation and snail control [8,21]. Also, the development of vaccines against schistosomiasis could be beneficial in control of schistosomiasis. However, despite promising results of some candidates in animal models, these candidates still have to be tested in human safety and efficacy trails and a vaccine is unlikely to become available before 2025 [22,23].

Fascioliasis

Fascioliasis in humans is a zoonotic parasitic disease caused by two liver fluke species: *Fasciola hepatica* and *Fasciola gigantica*. The final hosts of the parasites are mammals, mostly sheep and cattle, however, human infections are increasingly reported and the WHO estimated that worldwide at least 2.4 million people are infected [5]. Fascioliasis is of great veterinary importance and in tropical countries it is considered the most important helminth infection in cattle [6] causing economic damage by limiting productivity of livestock [5].

The life cycle of *Fasciola* species

The life cycle of *Fasciola* species involves a final host (a mammalian species), an intermediate host (a freshwater snail), and a carrier (a (semi-)aquatic plant) (Figure 2). Adult *Fasciola* reside in the bile ducts of their host where they feed primarily on blood and also on bile duct epithelium. In the bile duct, they produce immature eggs that are transported with bile into the intestine and subsequently excreted with faeces. In fresh water the eggs mature and a miracidium hatches from the egg. The miracidium will infect the intermediate host, a freshwater snail of the genus *Lymnaea*, in which it develops into sporocysts, rediae, and finally cercariae [7]. Cercariae leave the snail and will encyst on leaves of (semi-)aquatic plants, such as water cress, forming metacercariae that are protected by a tough cyst wall. Metacercariae can infect the definitive host when plants with attached metacercariae are consumed [5]. After ingestion, immature flukes excyst from the metacercaria in the small intestine [24]. Immature flukes will penetrate through the intestinal wall and migrate through the peritoneal cavity to the liver. They migrate through the liver and finally reach the bile duct [7]. In the bile duct the parasite becomes sexually mature and starts producing eggs [24]. Because the estimated life span of the adult parasite is between 9 and 13.5 years, infections may last for many years [5,7,25,26].

Symptoms of disease

Fascioliasis can be divided into two disease stages: acute hepatic fascioliasis and chronic hepatic fascioliasis. Acute fascioliasis is caused by migration of the juvenile flukes through the liver, resulting in symptoms such as fever, nausea and abdominal

pain. Usually eosinophilia is observed and the liver can be swollen [5,7]. Chronic fascioliasis arises when the adult parasites are established in the bile duct. This causes biliary obstruction resulting in symptoms such as intermittent pain and jaundice. Blood-feeding by the adult parasite results in the development of anaemia [5].

Diagnosis, treatment and control

Although eggs can be produced and secreted with faeces in chronic fascioliasis, detection of eggs in faeces is considered not a reliable diagnostic tool [27]. In the early stages of the disease, up to three to four months after infection, no eggs are produced yet and in chronic human fascioliasis egg shedding can be intermittent or very low [28]. Serological techniques are currently most often used for the diagnosis of fascioliasis. Anti-*Fasciola* antibodies can be detected in serum as early as two weeks after infection with ELISA. However, antibodies may persist for at least four to five months after successful treatment, making discrimination between past and present infection challenging [27]. Approximately eight weeks after infection, parasite antigens can be detected in serum or faeces of patients and could be used for diagnosis of active infection [27,28].

Triclabendazole is currently the drug recommended by the WHO in the treatment of fascioliasis [7]. Triclabendazole is effective against both the juvenile and adult stages of the parasite and the cure rate exceeds 90% after a single oral dose [7,29]. Although biliary colic is frequently observed after treatment, as a result of the passage of dead parasites through the bile duct, adverse events of triclabendazole are usually temporally and mild [29].

Control of human fascioliasis could be achieved by applying preventative chemotherapy using triclabendazole. Additionally, interruption of transmission through behavioural changes, snail control and treatment of infected livestock could attribute to control of human fascioliasis [5]. However, resistance against triclabendazole is emerging and forms a potential threat to control of human fascioliasis [7]. Vaccines for *F. hepatica* in livestock are under development, but despite the progress made, a vaccine against fascioliasis is still several years away [30–32].

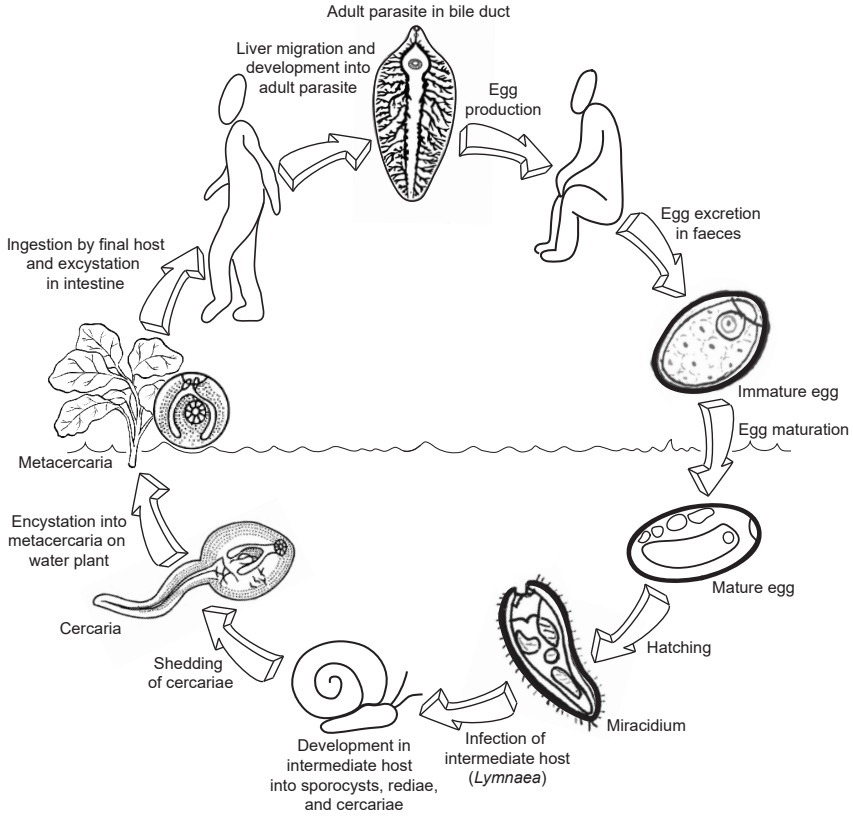

Figure 2. The life cycle of *Fasciola* species.

Host-parasite interactions in schistosomes

Schistosomes can reside in their human host for up to 30 years [8]. During this period they are under continuous attack by host defence mechanisms. The immune system and, since schistosomes are blood-dwelling parasites, the haemostatic system form the major sites of interaction between the host and the parasite. The interaction between schistosomes and host defence systems has been the focus of many past and ongoing studies.

The immune system

Immunology

During infection with schistosomes different stages can be discriminated, likewise, the immune response against the parasite can also be divided into three phases. During acute schistosomiasis, as the immature parasite migrates through tissues of its host, the dominant immune response is a Th1 response [33]. This response is characterized by the production of cytokines typical for a Th1 response, such as TNF, IFN-γ, IL-1, and IL-6 [33,34]. At around 6 weeks after infection, when the schistosomes have matured, mated, and started to produce eggs, the immune response shifts dramatically towards a strong Th2 response which peaks at 8 weeks after infection [33,35]. Specific soluble egg antigens that affect dendritic cells induce this shift toward a Th2 response, characterized by the production of IL-4, IL-5, IL-13, and anti-*Schistosoma* IgE [33,35]. Regulatory feedback mechanisms of Th2 responses, such as alternatively activated macrophages, IL-10, and regulatory T-cells, downregulate the Th2 response at around 12 weeks after infection [35]. This mild Th2 response characterized by IL-10 and regulatory T-cells is typical for the chronic stage of the infection.

Immune evasion strategies

Adult schistosomes reside in their host for many years and are, despite of an ongoing host immune response, not cleared. This clearly indicates that the parasite employs immune evasion strategies. The properties of the outer surface tegument are thought to play a crucial role in immune evasion. It has been proposed that high turnover of membranocalyx, the outer double membrane layer of the tegument, could be involved in rapid clearance of bound immune complexes or cells from the surface [10]. The membranocalyx could also function, similarly to a glycocalyx in bacteria, as protection against the activated complement system. Also, the acquisition of host molecules, such as MHC antigens, immunoglobulins, and low-density lipoprotein, to the schistosomal tegument could shield immunogenic epitopes and thereby prevent immune recognition of the worm [10]. Additionally, the surface acquisition of host decay accelerating factor, which prevents activation of complement, could further prevent complement activation at the parasitic surface [10]. Adaptive immune responses are also actively evaded by the adult worm. Schistosomal lipids, in particular lyso-phosphatidylserine, are responsible for the modulation of the Th2 response into a milder, regulated immune response in chronic schistosomiasis. Lyso-phosphatidylserine acts through Toll-like receptor 2 and polarizes dendritic cell maturation which could skew the adaptive immune system towards a Th2 response characterized by IL-10 and regulatory T-cells [36].

The haemostatic system

Principles of platelet plug formation, coagulation, and fibrinolysis

Haemostasis is a tightly regulated physiological mechanism that maintains blood in a fluid state in intact blood vessels and responds with coagulation at sites of injury to limit blood loss [37]. It consists of three elements: 1) platelet plug formation, 2) clot formation/coagulation, and 3) fibrinolysis.

Platelet plug formation, or primary haemostasis, at sites of injury is a first response in order to reduce blood loss. Damage of the endothelium exposes collagen fibres that form a binding site for the multimeric plasma protein von Willebrand Factor (VWF). Binding of VWF to collagen induces a conformational change in VWF, which results in exposure of platelet binding sites on VWF [38]. The interaction between collagen-bound VWF and platelets, slows the platelet down and allows interaction between collagen and platelets. Binding of platelets to collagen induces activation of platelets, resulting in release of two types of secretory granules, alpha and dense granules [37]. These granules contain a variety of molecules that amplify platelet activation, stimulate coagulation, induce vasoconstriction, and inhibit fibrinolysis. Platelet activation also leads to activation of the fibrinogen receptor, which allows binding of fibrinogen to platelets. The fibrinogen protein consists of two identical binding sites for platelets, which facilitates binding of fibrinogen to two different platelets resulting in platelet aggregation. Platelet aggregation is characterized by spreading of platelets and results in the formation of a platelet plug. Additionally, platelet activation also leads to the exposure of anionic phospholipids on platelets, which creates the optimal membrane surface to propagate coagulation activation [37,39].

The formed platelet plug needs to be stabilized in order to withstand forces caused by flowing blood [37]. The platelet plug is stabilized by a cross-linked network of fibrin fibres, thus converting the platelet plug into a clot. Fibrin is generated by cleavage of fibrinogen as a result of an orchestrated sequence of cleavage reactions of plasma coagulation factors, which is called secondary haemostasis or coagulation. Many of these plasma coagulation factors are primarily synthesised in the liver and impairment of liver function can have pronounced effects on blood coagulation [37].

The main activator of coagulation is the transmembrane protein tissue factor, which is present in the sub-endothelium and exposed upon vessel damage, but can also be expressed on activated endothelial cells and monocytes [37,40]. Tissue factor binds as a co-factor to activated coagulation factor VII (FVIIa), resulting in the formation of the TF-FVIIa complex [37]. In this complex, FVIIa can activate both FIX (at low tissue factor concentration) and FX (at high tissue factor concentration). FIXa will together with FVIII form the tenase complex, which activates FX to FXa. FXa forms together with FVa the prothrombinase complex which will convert prothrombin into thrombin, the enzyme

responsible for the conversion of soluble fibrinogen into insoluble fibrin. In addition to the tissue factor mediated activation pathway (intrinsic coagulation), high-molecular weight kininogen, kallikrein, and FXII can assemble on an anionic surface, resulting in the activation of FXI, which is called the contact activation pathway (extrinsic coagulation). FXIa can subsequently activate FIX, which is part of the tenase complex consisting of FIXa and FVIIIa. Thrombin is a key player in coagulation, as it converts fibrinogen monomers into an insoluble fibrin clot that is cross-linked by FXIIIa, thereby stabilizing the platelet plug. Additionally, thrombin is crucial in amplification of the coagulation cascade through activation of factors V and VIII which is essential for both the tenase and prothrombinase complex. Accumulation of thrombin over time induces anticoagulant mechanisms, that prevent uncontrolled clot formation, and induces antifibrinolytic pathways that ensure the clot persists long enough to allow initiation of tissue repair mechanisms.

Once tissue repair is initiated, the insoluble fibrin clot must be broken down in a process that is called fibrinolysis. This is initiated by the local secretion of tissue-type plasminogen activator (tPA) from the endothelium. tPA activates plasminogen by cleavage into plasmin, which directly cleaves fibrin into specific degradation products. Fibrin itself forms a crucial co-factor in activation of fibrinolysis by co-localizing enzyme and substrate. In the initial phase, tPA and plasminogen bind to binding sites on intact fibrin, which leads to slow activation of plasminogen to plasmin. Limited cleavage of fibrin by this low amount of plasmin will result in the exposure of additional binding sites (C-terminal lysines) that enhance both tPA and plasmin activity, resulting in a burst of plasmin that degrades the fibrin network and solubilizes the clot [37].

The haemostatic system is tightly regulated in order to prevent uncontrolled clot formation (anticoagulation) and allow the clot to persist long enough to allow endothelial damage to be repaired (antifibrinolysis). Anticoagulation is established through the actions of several peptidase inhibitors that inhibit coagulation factors, such as antithrombin, tissue factor pathway inhibitor, and activated protein C. Additionally, endothelial cells release nitic oxide and prostaglandins and express ecto-ADPase on their surface, which inhibit platelet activation and prevent coagulation in the absence of vessel damage. Also plasmin has anticoagulant functions as it is able to degrade many coagulation factors, thereby preventing further coagulation.

Key antifibrinolytic components are the serine protease inhibitors α2-antiplasmin and plasminogen activator inhibitor 1, that associate with the fibrin network and inhibit plasmin and tPA, respectively. Additionally, thrombin activatable fibrinolysis inhibitor acts to prevent a burst of plasmin activation after initial plasmin formation, by removal of C-terminal lysine binding sites from fibrin that would enhance tPA activity. Together, these mechanisms allow fine-tuning of haemostasis and prevent unwanted coagulation [37].

The haemostatic system and parasitic infections

Many blood-dwelling or blood-feeding parasites are expected to be potent activators of the haemostatic system. Their blood-feeding behaviour or presence in the vein could induce endothelial damage, provide a foreign surface, or could cause changes in blood flow, all of which normally activate haemostasis. However, the longevity of many parasite in their host indicates that parasites have developed strategies to modify activation of the haemostatic system and thereby survive the continuing interaction with haemostatic system of their human host.

Schistosomes

Adult schistosomes reside in the vasculature of their host for years, yet platelets do not adhere to the outer surface of the parasite [41] and thrombotic complications are relatively uncommon in hepatosplenic schistosomiasis patients (5% of hepatosplenic patients) [42]. Therefore, schistosomes are proposed to have numerous mechanisms that impede clot formation and promote the degradation of blood clots that do form, which will be described in detail elsewhere in this thesis. In contrast, binding of VWF [43] and massive platelet adhesion and aggregation is observed on the surface of schistosome eggs [41]. It is suggested these capacities of the eggshell are important for anchoring eggs to the vessel wall and subsequent egg extravasation [41,43].

Despite modification of the haemostatic system by adult schistosomes, abnormalities in haemostasis are common in patients suffering from chronic hepatosplenic schistosomiasis [44]. In particular, bleeding from gastro-oesophagal varices is a common fatal complication in these patients. Haemostatic abnormalities observed in these patients are linked to reduction in liver function, as a result of granuloma formation around trapped eggs, and include: reduced levels of both plasma coagulation and anticoagulation factors, lower numbers of platelets, impaired coagulation *in vitro*, increased thrombin production, and enhanced fibrinolysis [44]. Thus, in schistosomiasis both local effects on haemostasis by the adult parasite and/or eggs, and systemic effects on haemostasis as a result of liver pathology are present.

Other blood-feeding or blood-dwelling parasites

In addition to schistosomes, many other blood-feeding or blood-dwelling parasites interact with the human haemostatic system and have developed their own strategies to survive. Some examples will be discussed below.

Malaria is the most important disease-causing parasite in humans and its life cycle is characterized by a blood stage that causes the characteristic fever symptoms of malaria. In this stage, the parasite infects red blood cells and promotes binding of these cells to the vessel wall, to avoid clearance of infected cells by the spleen. Haemostatic changes are observed during malaria infection, which includes: increased levels of

VWF, reduced activity of the VWF-cleaving peptidase ADAMTS13, enhancement of extrinsic and intrinsic coagulation, and attenuation of anticoagulant mechanisms [45]. Accumulation of VWF during infection is suggested to facilitate binding of infected red blood cells to the vascular wall. The strong coagulation activation observed during malaria has been suggested to play a role in malaria pathogenesis, however, currently it is unclear whether coagulation activation actually modulates malaria pathogenesis or whether it is a consequence of infection [45].

Also in lymphatic filariasis part of the parasitic life cycle is located in the human bloodstream. While the adults reside in the lymphatic system, the microfilariae they produce enter the blood vessels. Microfilariae have been described to inhibit collagen and ADP-induced platelet aggregation, possibly through secretion of anti-aggregatory eicosanoids, and the parasite secretes an inhibitor of coagulation factor XII, which may prevent coagulation initiation [46,47].

Not only blood-dwelling parasites but also blood-feeding parasites must produce anticoagulants in order to allow prolonged periods of blood-feeding. Adult *Fasciola* can reside in the bile duct for over a decade and clear changes in host haemostasis are observed during infection. The parasite seems to interfere with ATP, ADP, and AMP hydrolysis, which could affect platelet aggregation during infection [48]. Additionally, excretory/secretory products of *F. hepatica* are shown to accelerate intrinsic blood coagulation, while the extrinsic coagulation pathway is delayed, however, the mechanisms underlying these coagulation changes and its effect on pathogenesis need to be further elucidated [49]. Furthermore, examination of the components of the excretory/secretory products of *F. hepatica* showed the presence of host-derived products, including antithrombin and α-2-macroglobulin, that are involved in regulation of the haemostatic system [50]. Re-use of host-derived inhibitors may thus form another strategy to modify host coagulation.

Not only internal blood-feeding parasitic worms combat the haemostatic system. Blood-feeding ectoparasites, such as mosquitos, ticks, and leeches have also been described to contain inhibitors directed at different stages in the coagulation cascade. Especially leeches are interesting, since one well-known antithrombotic agent, hirudin, has been isolated from medicinal leeches [51]. Hirudin is a potent inhibitor of thrombin. However, this is not the only anticoagulant protein that leeches secrete [52]. Leeches are well adapted to their host haemostatic system and have a broad arsenal of agents that increase blood flow, inhibit platelets, and have anticoagulant properties, to ensure blood-feeding for prolonged periods [52].

Aim and outline of this thesis

The focus of this thesis is on the interaction of parasitic helminths, in particular schistosomes, with the blood coagulation system of their host. Prolonged presence of the adult schistosome pair in the vasculature is expected to activate the blood coagulation system. The interaction of schistosomes with blood coagulation has been investigated by many other researchers [41,53,62–71,54,72–75,55–61]. Also, coagulopathy that is observed after prolonged (hepatosplenic) infection is described in detail (reviewed by Tanabe *et al.* [44]). However, so far a complete overview of the mechanisms schistosomes utilize to interfere with human haemostasis is missing and it is unknown whether there are any yet undiscovered strategies the parasite applies for evading blood coagulation. Furthermore, insights in the presence of coagulopathy in non-hepatosplenic schistosomiasis is currently not available. The aim of the work in this thesis is to (1) present an overview on the blood coagulation modification mechanisms of schistosomes, (2) provide insight in coagulopathy in schistosome infections without hepatic disease, and (3) identify yet unknown mechanisms of modification of blood coagulation by schistosomes and other blood-feeding parasites.

Schistosomiasis: interactions with the haemostatic system and haemostatic changes during infection

As the adult schistosome worm pair resides in the human vasculature for prolonged periods, this forms a potential prothrombotic threat. Activation of coagulation is negative for both the host, leading to thrombosis, and the parasite as coagulation around the parasite can restrict movement of the worm pair. It is therefore clear that schistosomes must counteract the activation of blood coagulation. So far there is no complete overview on the mechanisms that are used by schistosomes to modify blood coagulation. In **Chapter 2**, we have summarized the studies on the interactions between schistosomes and the host haemostatic system to present a comprehensive overview of the mechanisms currently known. This literature overview also showed us that, although much research has been performed on coagulopathy in schistosomiasis, most studies have been performed in hepatosplenic schistosomiasis patients. Many coagulation factors are synthesised in the liver, and it is therefore difficult to discriminate between the effects of the schistosome infection and the liver damage on blood coagulation of the host. In **Chapter 3**, we performed a pilot study aimed at unravelling the contribution of solely the parasitic infection on changes in blood coagulation by studying coagulation parameters in a group of Gabonese school children suffering from urogenital schistosomiasis haematobium. As mentioned above, adult *S. haematobium* reside in the perivesicular veins, so infection

with this species does usually not result in liver damage. This allowed us to determine the specific effects of the parasitic infection only, and showed that inflammation mediated endothelial activation results in increased plasma VWF levels, without increased activation of coagulation in these patients.

Alternative pathways to regulation of VWF function

The finding that plasma VWF levels are increased in *S. haematobium* patients without apparent activation of coagulation, led us to further investigate the interaction between VWF and adult schistosomes. Interactions between VWF and schistosome eggs have already been studied thoroughly [43]. This revealed that VWF binds directly to the egg shell and this is essential for the adhesion of the eggs to the endothelium, leading to extravasation of the eggs from the circulation. The interactions between adult schistosomes and VWF have not been investigated before, but the increased plasma VWF levels during infection, without coagulation activation, suggests that the adult parasite has evolved mechanisms to regulate VWF function. **Chapter 4** describes the discovery of a *S. mansoni* peptidase that is capable of cleaving both a substrate that is used to measure VWF-cleavage, FRETS-VWF73, and purified VWF. This peptidase belongs to the class of cysteine peptidases and is inhibited by the peptidases inhibitors N-ethylmaleimide and leupeptin, but activated by the chelating agent ethylenediaminetetraacetic acid (EDTA) or the reducing agent L-cysteine. Using a combination of anion exchange chromatography and mass spectrometry the *S. mansoni* cathepsin B2 gene, SmCB2, was identified as the prime candidate to encode the *S. mansoni* VWF-cleaving peptidase. Although reduction of SmCB2 expression in adult worms by RNA interference did not affect the proteolytic activity towards VWF, recombinant expressed SmCB2 (rSmCB2) could cleave the FRETS-VWF73 substrate, which contains the A2 domain of VWF. This confirmed that SmCB2 is a schistosomal cysteine peptidase that can cleave human VWF.

After investigating alternative pathways to regulate VWF functionality during schistosomiasis, we next focussed on alternative pathways for regulation of VWF functionality during normal haemostasis. Direct cleavage of VWF by peptidases other than ADAMTS13 has been described in several studies [76–81]. However, to date regulation of ADAMTS13 is still unknown. Several studies suggest that the fibrinolytic enzyme plasmin can cleave and thereby inactivate ADAMTS13. In **Chapter 5**, we found that plasmin is capable of inducing truncation of ADAMTS13, leading to increased activity of this peptidase. Plasmin may thus form a physiological mechanism to enhance ADAMTS13 activity.

S. mansoni and the fibrinolytic system

Plasmin also forms a key player in the fibrinolytic system, where it functions in the breakdown of formed thrombi. Many studies have been performed on activation of plasminogen into plasmin by schistosomes [71–75]. These studies show that schistosomes have the capacity to bind and enhance activation of plasminogen to plasmin, however, the plasminogen activator tissue-type plasminogen activator (tPA) is required for the enhancement of plasminogen activation. The interaction between schistosomes and tPA is therefore the subject of **Chapter 6**. In this study we investigated if schistosomes have proteins that can bind tPA, especially at the host-parasite interface, which could aid in the enhancement of plasminogen generation by schistosomes.

Cleavage of coagulation factors by *F. hepatica*

Not only blood-dwelling parasites interact with blood coagulation, also blood-feeding parasites have to modify blood coagulation to allow blood-feeding for prolonged periods. We therefore investigated the effect of several secreted peptidases of the blood-feeding parasites *F. hepatica* and *S. mansoni* for effects on proteins involved in coagulation in **Chapter 7**. This led to the discovery that cathepsin L peptidases of *F. hepatica* cleave fibrinogen and fibrin. We suggest that this could aid in preventing blood coagulation in the parasite gut, thereby allowing blood-feeding for extended periods.
The conclusions and implications of these studies are summarized and discussed in **Chapter 8.**

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2

CHAPTER 2

Interference with the host haemostatic system by schistosomes

Mirjam M. Mebius[1], Perry J. J. van Genderen[2], Rolf T. Urbanus[3], Aloysius G. M. Tielens[1], Philip G. de Groot[3], Jaap J. van Hellemond[1]

[1] Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center Rotterdam, The Netherlands
[2] Department of Internal Medicine and Institute of Tropical Diseases, Harbor Hospital Rotterdam, The Netherlands
[3] Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, The Netherlands

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Abstract

Schistosomes, parasitic flatworms that cause the tropical disease schistosomiasis, are still a threat. They are responsible for 200 million infections worldwide and an estimated 280,000 deaths annually in sub-Saharan Africa alone. The adult parasites reside as pairs in the mesenteric or perivesicular veins of their human host, where they can survive for up to 30 years. The parasite is a potential activator of blood coagulation according to Virchow's triad, because it is expected to alter blood flow and endothelial function, leading to hypercoagulability. In contrast, hepatosplenic schistosomiasis patients are in a hypocoagulable and hyperfibrinolytic state, indicating that schistosomes interfere with the haemostatic system of their host. In this review, the interactions of schistosomes with primary haemostasis, secondary haemostasis, fibrinolysis, and the vascular tone will be discussed to provide insight into the reduction in coagulation observed in schistosomiasis patients.

Interference with the haemostatic system by pathogens is a common mechanism and has been described for other parasitic worms, bacteria, and fungi as a mechanism to support survival and spread or enhance virulence. Insight into the mechanisms used by schistosomes to interfere with the haemostatic system will provide important insight into the maintenance of the parasitic life cycle within the host. This knowledge may reveal new potential anti-schistosome drug and vaccine targets. In addition, some of the survival mechanisms employed by schistosomes might be used by other pathogens, and therefore, these mechanisms that interfere with host haemostasis might be a broad target for drug development against blood-dwelling pathogens. Also, schistosome antithrombotic or thrombolytic molecules could form potential new drugs in the treatment of haemostatic disorders.

Introduction

The haemostatic system consists of procoagulant and anticoagulant mechanisms that stop bleeding at sites of blood vessel injury and play an important role in innate immunity [1-3]. Procoagulant mechanisms of the haemostatic system can be further divided into primary and secondary haemostasis. Primary haemostasis involves the activation and aggregation of blood platelets, whereas secondary haemostasis involves a cascade of proteolytic reactions that lead to the formation of a stable fibrin clot. Anticoagulant mechanisms of the haemostatic system include inhibitors of primary and secondary haemostasis and the fibrinolytic activity of plasmin that leads to degradation of formed fibrin clots [2]. According to Virchow's triad, three conditions can contribute to the initiation of blood coagulation: normal blood flow is disrupted or altered (stasis); the endothelium is damaged or dysfunctional; and/or the coagulability of blood plasma is increased (hypercoagulability) [4-6]. In order to maintain and propagate themselves in blood vessels, many blood-dwelling pathogens not only require adaptations to evade the actions of the host immune system but also need to avoid blood coagulation through interference with the haemostatic system of their host. Schistosomes, blood-dwelling parasitic flatworms, are the cause of the tropical disease schistosomiasis [7]. On average, adult schistosomes reside in their host's bloodstream for three to five years, but their individual lifespan can be as long as 30 years [7]. Schistosomes can be expected to activate coagulation according to Virchow's triad by inducing stasis and alterations in endothelial function [8,9]. The adult schistosome pair disturbs blood flow due to the large size of the worm pair: 1 cm long with a diameter of 1 mm (Figure 1). Light microscopy images of adult worms inside the mesenteric veins showed that the worm pair occupies the major part of the lumen of the blood vessels in which they reside [8,10]. This obstruction will induce turbulence in the vein and increase shear stress along the vessel wall. Turbulence has been described to contribute to the formation of thrombi [11]. Furthermore, endothelial cells can be activated by oscillatory blood flow, which is characterized by forward–reverse flow cycles and disrupted blood flow downstream of sites where the vessel lumen is narrowed [12]. This leads to increased expression of molecules involved in blood coagulation and modulation of the vascular tone, such as tissue factor (TF), von Willebrand Factor (VWF), tissue-type plasminogen activator (t-PA), nitric oxide (NO), and prostacyclin (PGI_2) [13-18]. Turbulence and changes in shear stress, induced by the presence of the adult schistosome pair in the blood vessel, could potentially activate platelets and blood coagulation [4,11]. In addition, although there is no direct evidence of endothelial damage caused by the presence of the adult worm pair in the vein, several studies suggest that schistosomes disturb endothelial cell function, and it has been suggested that the presence of the adult worm in the

vein induces endothelial damage [9,19-21]. In murine schistosomiasis, the expression of endothelial NO synthase as well as the production of NO are decreased, which indicates endothelial dysfunction [9,19,21]. Furthermore, plasma soluble intercellular adhesion molecule-1 is increased in hepatosplenic schistosomiasis patients, which indicates endothelial activation and inflammation [22]. Extravasation of schistosome eggs may also contribute to endothelial damage or dysfunction, since this disrupts the polarization of the endothelium and causes mobilization and migration of endothelial cells [8]. Therefore, it is likely that parasite-induced alteration in endothelial function or endothelial damage plays a role in activation of blood coagulation. Besides alterations in blood flow and endothelial function, schistosomes have many electronegative charges on their surfaces that could potentially activate platelets and the coagulation cascade, leading to hypercoagulation [23]. Thus, schistosomes have all the characteristics to be potent activators of blood coagulation. However, schistosomiasis patients do not have an increased risk of thrombus formation [24]. In contrast, studies on blood coagulation in hepatosplenic schistosomiasis patients (reviewed by Tanabe [24]) showed that patients have prolonged coagulation times [25]. In infected humans, major haemostatic abnormalities are only observed in hepatosplenic schistosomiasis patients, but murine studies observed changes in the activity of several coagulation factors already during the early phase of schistosomiasis [26]. Hepatosplenic schistosomiasis patients have a reduced activity or reduced levels of the coagulation factors II, VII, IX, X, XI, XII, fibrinogen, high–molecular-weight kininogen (HMWK), and prekallikrein, as well as the regulatory proteins antithrombin and protein C [27,28]. Furthermore, the levels of thrombin-antithrombin complexes, prothrombin fragment 1+2, plasma fibrinopeptide A, D-dimers, and other fibrin degradation products are increased in these patients [25,29]. The elevated levels of both markers of coagulation activation (e.g., prothrombin fragment 1+2 and plasma fibrinopeptide A) as well as markers of fibrinolysis (e.g., fibrin degradation products) indicate a continuous activation of both blood coagulation and fibrinolysis in hepatosplenic schistosomiasis patients. Therefore, the observed hypocoagulable and hyperfibrinolytic state of these individuals is the result of both increased consumption of coagulation factors and decreased hepatic synthesis of these factors and cannot solely be attributed to hepatic dysfunction [24,29]. Also, research showed that blood platelets do not adhere to adult schistosomes or isolated outer surface membranes (tegument) of adult worms [30]. It is thus clear that schistosomes must have mechanisms that suppress the haemostatic response of their host. In this review, the interactions of schistosomes with primary haemostasis, secondary haemostasis, fibrinolysis, and the vascular tone will be discussed in order to provide insight into the reduction in blood coagulation that is observed in schistosomiasis patients.

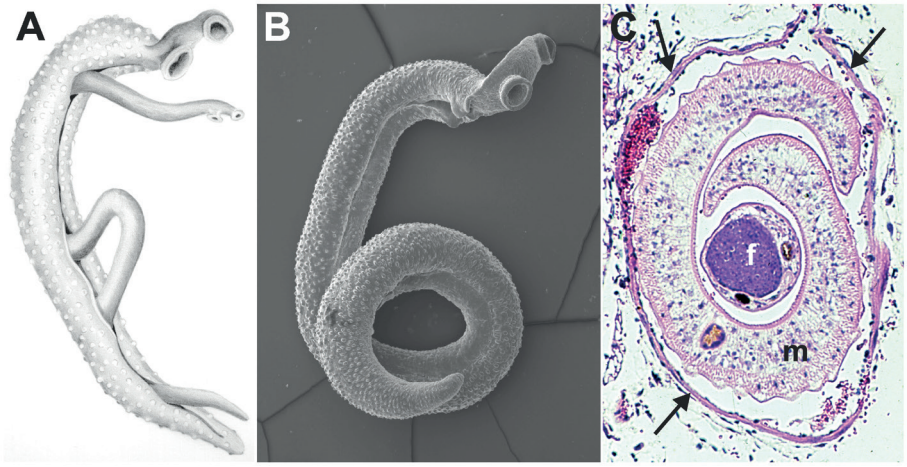

Figure 1. Images of adult schistosomes.

Panel A shows a schematic drawing of an adult worm pair. The large adult male embraces the smaller female worm and both worms have two suckers by which they attach to the blood vessel wall. Panel B shows a scanning electron microscope image of a single *S. mansoni* adult male, which is about 1 cm long with a diameter of 1 mm. Panel C shows a cross-section of an adult *S. mansoni* worm pair (m, male; f, female; arrows mark the vessel wall) in a mesenteric venule of a mouse. This cross-section illustrates how close the worm pair is to the vessel wall and suggests the extent to which the worms must disturb blood flow (Panel C is adapted from D. G. Colley and W. E. Secor, PLoS Neglected Tropical Diseases 2007 [10]).

Identification of schistosome mechanisms that interfere with the haemostatic system provides important insight into the maintenance of the parasitic life cycle within its host. Insight into survival mechanisms of the parasite could provide important clues for novel anti-schistosome drugs or reveal vaccine targets. In addition, other blood-dwelling pathogens face similar survival challenges and may therefore employ similar survival strategies as schistosomes. These mechanisms that interfere with host haemostasis may, therefore, form a broad target for drug development against blood-dwelling pathogens. Also, potent antithrombotic drugs that are currently used in the clinic have been isolated earlier from pathogens, such as streptokinase from *Streptococci* [31]. Schistosome antithrombotic or thrombolytic molecules could therefore form potential novel drugs in the treatment of haemostatic disorders.

Interference with primary haemostasis by schistosomes

Primary haemostasis consists of the activation and aggregation of blood platelets. Platelet activation can be triggered by endothelial damage, which leads to exposure of the underlying collagen, or by the presence of soluble activators, such as thrombin or ADP. When the vessel wall is damaged, platelets will adhere to collagen-bound VWF through glycoprotein Ib (GPIb) present on their surface, followed by their

activation and degranulation. Under pathophysiological conditions VWF also binds to surfaces of pathogens, such as *Staphylococcus aureus,* and triggers platelet activation [32]. Activated platelets release factors, such as ADP and thromboxane A2, which induce vasoconstriction, stimulate secondary coagulation, and promote further platelet activation and aggregation, resulting in the formation of a stable platelet plug [2]. Several mechanisms have been described that could explain the ability of schistosomes to prevent primary haemostasis (Figure 2).

Ngaiza and Doenhoff observed a decreased platelet count, also called thrombocytopenia, during schistosome infection in mice [33]. This was suggested to contribute to the observed decrease in platelet aggregation around the adult schistosome pair. In schistosomiasis patients, a decreased platelet count was also observed, but symptoms that are commonly observed in patients with thrombocytopenia, e.g., gingival bleeding, are not present in these patients, indicating that platelet aggregation is not fully impaired [34]. Schistosomes must therefore have evolved additional mechanisms to prevent primary haemostasis.

Extracellular ADP induces platelet aggregation. ADP-mediated platelet aggregation is normally controlled by ATP-diphosphohydrolase (ATPDase) proteins, such as CD39/ATPDase1, that are present on endothelial cells [35,36]. These ATPDases hydrolyse ATP to ADP and ADP to AMP and subsequently to adenosine. This degradation of ADP and the subsequent formation of the inhibitor adenosine leads to inhibition of ADP-mediated platelet activation and aggregation [37,38]. The schistosome tegument contains several enzymatic activities that could lead to the degradation of extracellular ATP or ADP [39]. Alkaline phosphatase activity is present in the tegument of *Schistosoma mansoni,* and recombinant *S. mansoni* alkaline phosphatase (SmAP) has been characterized [40,41]. Alkaline phosphatase enzymes are present in many organisms and hydrolyze a broad spectrum of substrates, including ATP, ADP, and AMP [42]. *S. mansoni* alkaline phosphatase (SmAP) has structural homology to human placental alkaline phosphatase, which suggests that its substrate specificity is similar to human alkaline phosphatases. In addition, investigation of the *S. mansoni* tegument revealed phosphodiesterase (SmPDE) activity, which hydrolyzes, among others, ATP and AMP [43]. Furthermore, a tegument-localized *S. mansoni* ATP-diphosphohydrolase-1 (SmATPDase1) activity was characterized, which is capable of hydrolysis of both ATP and ADP to AMP [44,45]. Therefore, these tegumental enzymes could, by mimicking human ATPDases, form a potential strategy to inhibit platelet activation.

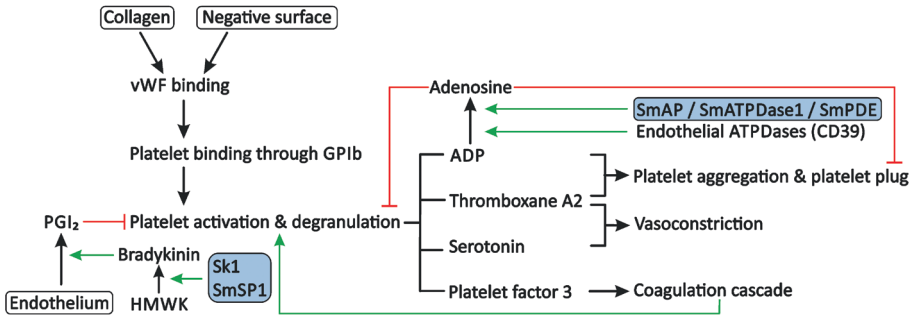

Figure 2. Proposed modulation of primary haemostasis by schistosomes.

Primary haemostasis consists of the activation of blood platelets and the formation of a platelet plug. Exposure of subendothelial collagen, due to endothelial damage or the presence of soluble activators or surfaces of pathogens, can facilitate binding of VWF. Platelets adhere to VWF through GPIb present on their surface, followed by their activation and degranulation. Platelets release molecules, such as ADP, thromboxane A2, serotonin, and platelet factor 3, which stimulate further platelet aggregation and activation, stimulate vasoconstriction, and activate secondary haemostasis. Primary haemostasis is inhibited by PGI_2 from endothelial cells, which inhibits degranulation of platelets, and by the degradation of extracellular ADP to AMP and subsequently to the inhibitor adenosine by endothelial ATPDases, such as CD39. Schistosomes interfere with the primary haemostasis through the proposed degradation of extracellular ADP by SmAP, SmATPDase1, and/or SmPDE, and potentially stimulate the release of PGI_2 through up-regulation of the production of bradykinin by sK1 or SmSP1. Stimulation is indicated by green arrows. Red lines indicate inhibition. Schistosome proteins are indicated in the shaded boxes. Abbreviations: glycoprotein Ib (GPIb), High-molecular-weight kininogen (HMWK), prostacyclin (PGI_2), *Schistosoma mansoni* alkaline phosphatase (SmAP), *Schistosoma mansoni* ATP-diphosphohydrolase-1 (SmATPDase1), *Schistosoma mansoni* phosphodiesterase (SmPDE), von Willebrand Factor (VWF).

Furthermore, the actions of an *S. mansoni* protein with kallikrein-like activity, sK1, and a protein with homology to mouse plasma kallikrein, SmSP1, may form a mechanism through which schistosomes inhibit primary haemostasis [46,47]. Kallikrein is able to convert HMWK to the small vasoactive peptide bradykinin, which is a potent vasodilator [20,48]. Bradykinin can trigger the release of prostacyclin (PGI_2) from endothelial cells [48]. PGI_2 is another potent vasodilator but also inhibits platelet degranulation. Exposure on the surface or secretion of the tegumental proteins sK1 and SmSP1 has been suggested, and it has been shown that sK1 is able to convert HMWK to bradykinin *in vitro*, which suggests the production of bradykinin by these proteins also during schistosome infection *in vivo* [46,47]. Kallikrein-like activity has not been described for SmSP1, but its homology to mouse kallikrein suggests kallikrein-like activity. sK1, and potentially SmSP1, could thus play a role in the inhibition of primary haemostasis by the parasite, although such a role has not been demonstrated.

Interference with secondary haemostasis by schistosomes

Secondary haemostasis consists of two pathways: the tissue factor (TF) pathway (extrinsic pathway) and the contact activation (intrinsic) pathway [2,49]. The extrinsic pathway is considered to be the physiologically most relevant pathway and is induced by the exposure of TF after endothelial damage, but under pathophysiological conditions it can also be activated by TF expressed on immune cells or endothelial cells [3,50]. The intrinsic pathway is activated by binding of coagulation factor XII (XII) to collagen or negatively charged surfaces [51,52]. Local accumulation of XII leads to its auto-activation and induces a cascade of cleavage reactions that activate other coagulation factors [53]. Finally, both the extrinsic and intrinsic pathways lead to the cleavage of fibrinogen to fibrin by thrombin and the formation of a stable fibrin clot (Figure 3).

Interference with the activation of secondary haemostasis by schistosomes

Schistosomes are potential activators of the extrinsic pathway due to the induction of turbulence and endothelial damage and/or alteration of endothelial function induced by the adult parasite and/or the eggs [8,9,16]. Furthermore, elevated levels of tumor necrosis factor alpha (TNF-α) are present in schistosomiasis patients, and high TNF-α levels are known to induce TF expression on endothelial cells *in vitro* [50,54]. In addition, increased TF expression is present in monocytes from hepatosplenic schistosomiasis patients, compared to monocytes of healthy donors, suggesting the involvement of monocyte TF expression in the prothrombotic state observed in schistosomiasis patients [54]. However, to date there is no evidence that schistosomes interfere with extrinsic coagulation [55].

On the other hand, *in vitro* experiments show that schistosomes can inhibit the activation of secondary haemostasis in at least two steps: the activity of thrombin and the activation and activity of XII, a factor of the intrinsic pathway, suggesting inhibition of secondary haemostasis during schistosome infection *in vivo* [55-58]. Both the extrinsic and the intrinsic pathway lead to the activation of thrombin. The inhibition of thrombin activity by schistosomes could therefore be a potential strategy to prevent the formation of fibrin clots. The *S. mansoni* antigen Sm22.6 discovered by Stein and David is expressed in the cytoplasmic layer of the schistosome tegument but is also present in the host circulation [59]. The exact role of Sm22.6 in the parasitic life cycle is unknown, but the protein interacts with thrombin and inhibits its protease activity [58]. This suggests that Sm22.6 could prevent the formation of fibrin clots around the adult parasite [58]. Furthermore, thrombin plays an important role in the amplification loop of the coagulation cascade through the activation of XI by thrombin; blocking this protein could interfere with this positive feedback mechanism.

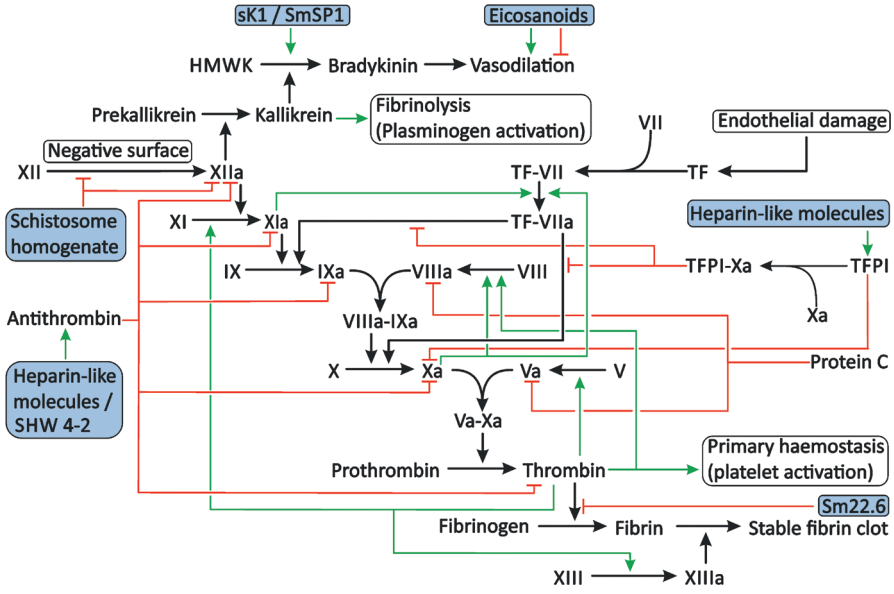

Figure 3. Proposed modulation of secondary haemostasis and vascular tone by schistosomes.

Secondary haemostasis can be activated through two different pathways: either by the presence of TF or by contact of coagulation factors with collagen, pathogens, or other negatively charged surfaces. This triggers a cascade of cleavage reactions, ultimately leading to the cleavage of fibrinogen to fibrin and the formation of a stable fibrin clot. This process is regulated by antithrombin, TFPI, and protein C. Schistosomes potentially interfere with secondary haemostasis at several steps in the cascade. Schistosome whole worm homogenate blocks the conversion of XII to XIIa and inhibits the actions of XIIa. The proteolytic activity of thrombin is inhibited by the schistosome antigen Sm22.6. Furthermore, schistosome heparin-like glycosaminoglycans may enhance the activity of antithrombin and, possibly, TFPI, and the schistosome serpin SHW 4-2 might mimic human antithrombin. The vascular tone can be influenced by schistosomes through the production of both vasodilating and vasoconstricting eicosanoids and the presence of sK1 and SmSP1 that could potentially convert HMWK into the vasodilator bradykinin. The green arrows indicate stimulation. Inhibition is indicated by the red lines. The shaded boxes indicate schistosome proteins. Abbreviations: activated coagulation factor XII (XIIa), coagulation factor XII (XII), High-molecular-weight kininogen (HMWK), tissue factor (TF), tissue factor pathway inhibitor (TFPI).

Schistosomes have also been observed to interfere with the intrinsic pathway. Whole worm homogenate of *S. mansoni* inhibits both the activation and activity of XIIa [55-57]. The molecules responsible for this inhibitory activity have not been characterized, and their localization in the adult worm is, therefore, not known. Further investigations are required to confirm this suggested activity. Furthermore, the importance of XII in coagulation *in vivo* is still debated [60]. XII deficiency does not result in a bleeding tendency *in vivo,* in contrast to a decreased coagulation time observed in *in vitro* tests [61]. The reason for this discrepancy remains unclear, but it led to the assumption that XII is not required for normal haemostasis, although a role has been suggested in pathological thrombus formation [60]. It should therefore be stressed that the role

of inhibition of XII in prevention of coagulation during schistosome infections might be very limited. Another possibility could be that inhibition of XII by schistosomes is not involved in interference with haemostasis, but instead functions in the evasion of immune responses by the parasite. XII is involved in the activation of the complement cascade, and inhibition of XII by schistosomes might thus be an example of an immune-evasion strategy rather than a strategy to prevent blood coagulation [62].

Interference with regulatory mechanisms of secondary haemostasis by schistosomes

Secondary coagulation is regulated by the actions of three proteins: antithrombin, tissue factor pathway inhibitor (TFPI), and protein C. Stimulation or mimicking of host mechanisms for the regulation of blood coagulation could form a potential strategy employed by schistosomes to interfere with haemostasis.

The serine protease inhibitor (serpin) antithrombin is the major inhibitor of coagulation proteases [61]. Antithrombin is constitutively present in blood plasma and binds and inactivates thrombin, as well as coagulation factors IXa, Xa, and XIa. The efficiency of thrombin binding increases 2,000- to 10,000-fold when antithrombin associates with its cofactor heparin [61]. Schistosomes could potentially increase antithrombin activity by expressing heparin or heparin-like molecules, such as heparan sulfate. Heparan sulfate is a heparin-like molecule that is present on the surface of endothelial cells and is responsible for the anticoagulant properties of the endothelium. Heparin-like glycosaminoglycans, i.e., heparan sulfate and dermatan sulfate, are present in tegument fractions of *S. mansoni* [63]. However, the localization of these heparin-like molecules on the outer tegumental membrane and the capacity of these molecules to interfere with the haemostatic system have not been determined.

Furthermore, Blanton *et al.* identified a serpin in *S. haematobium*, SHW 4-2, with high sequence similarity to antithrombin and glial-derived nexins, which can both bind and inhibit thrombin [64,65]. Surface localization of SHW 4-2 was shown by immunolocalization, suggesting that *S. haematobium* serpin may mimic the actions of antithrombin and could play a role in the inhibition of secondary haemostasis.

TFPI, the natural regulator of TF activity, is present in blood plasma and platelets, and is synthesized by endothelial cells. Its mode of action is peculiar; TFPI binds and inhibits coagulation factor Xa. This TFPI-Xa complex subsequently binds to the TF-VIIa complex, thereby inhibiting further activity of coagulation factor VIIa. TFPI present in plasma is active, but present in low concentrations and only able to delay the coagulation cascade. TFPI can be released by degranulation of activated platelets and/or from the endothelium by heparin and thereby regulate TF activity [2,66]. The presence of heparin-like molecules on the outer surface of adult schistosomes suggests that the parasite may stimulate the release and local accumulation of TFPI from the endothelium, although this has not been confirmed.

Stimulation of fibrinolysis by schistosomes

Besides interference with primary or secondary haemostasis, schistosomes may also reduce thrombus formation through stimulation of fibrinolytic pathways. Fibrinolysis by plasmin controls the degradation of fibrin clots to fibrin degradation products (Figure 4). The proteolytic activation of plasminogen to plasmin is stimulated by plasma kallikrein, but mainly by urokinase and t-PA, which are slowly released from damaged endothelium [2,67]. Although several reports show no enhancement of fibrinolysis by *S. mansoni* [55,56], more recent reports indicate the presence of activators of plasminogen at the surface of *S. bovis* [68-70]. Ramajo-Hernández *et al*. screened tegument fractions for proteins binding plasminogen and demonstrated that tegument fractions enhanced the generation of plasmin by t-PA [68]. Ten proteins that were able to bind plasminogen were identified; the most prominent ones were enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and actin. Surface localization was shown for GAPDH and enolase but not for actin [69,71]. Enolase is expressed on the surface of male schistosomes only, and recombinantly expressed enolase has been shown to bind plasminogen and enhance its conversion to plasmin in the presence of t-PA [68,69]. Similarly, Yang *et al*. discovered a plasminogen-binding enolase in *S. japonicum*, indicating that plasminogen-binding by schistosome enolases may be a common feature of schistosomes [72]. The role of GAPDH and actin in the conversion of plasminogen is still unclear, but it has been described that interaction of plasminogen with other molecules induces conformational changes in plasminogen which could aid in the conversion of plasminogen to plasmin by t-PA [73]. Further research by de la Torre-Escudero *et al*. revealed on the parasitic surface the presence of a schistosome protein belonging to the family of annexins [70]. Annexins have many functions, and some annexins, such as human annexin-A2, are involved in the regulation of fibrinolysis [74]. Schistosome annexin binds plasminogen and enhances the t-PA mediated conversion of plasminogen to plasmin [70]. The schistosome surface annexin may thus have a role in local activation of fibrinolysis during schistosome infection, thereby stimulating the degradation of thrombin clots that could be formed at the parasite's surface.

Interference with the vascular tone by schistosomes

Besides a possible activation of blood coagulation by the parasite, the parasite faces a second problem in the bloodstream. The eggs deposited by the female are laid in the small veins surrounding the intestine, and the parasite pair is known to wander between the large portal vein and these small veins [75]. This migration could, due to the large size of the adult worm pair compared to the size of the blood vessels, temporarily cause obstruction of blood flow, and the resulting changes in blood flow could potentially damage the endothelium and surrounding tissues. Manipulation of the vascular

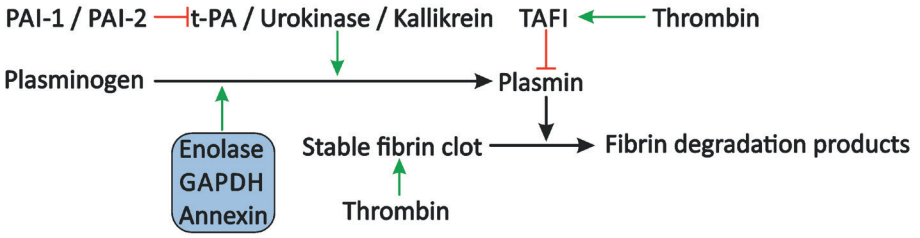

Figure 4. Proposed modulation of the fibrinolytic system by schistosomes.

The fibrinolytic system inhibits blood coagulation through degradation of the formed fibrin clot by plasmin. Plasminogen is cleaved to plasmin by plasma kallikrein, but mainly by t-PA and urokinase, which are inhibited by PAI-1 and PAI-2. Furthermore, plasmin activity is inhibited by TAFI. Schistosomes may stimulate fibrinolysis by the presence of enolase, GAPDH, and annexin, which bind plasminogen and facilitate its conversion to plasmin by t-PA. Green arrows indicate stimulation. Inhibition is indicated by the red lines. Schistosome proteins are denoted in the shaded box. Abbreviations: tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor 1 (PAI-1) and 2 (PAI-2), thrombin-activatable fibrinolysis inhibitor (TAFI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

tone, e.g., by inducing vasodilation, could prevent damage caused by obstruction of these small blood vessels, and such a strategy would therefore be advantageous for the parasite. Several mechanisms have been proposed by which schistosomes could modulate vascular tone (Figure 3) (reviewed in detail by Da'dara and Skelly [20]). For example, schistosomes are able to produce and secrete eicosanoids. Eicosanoids have diverse functions; among these is influencing the vascular tone. *S. mansoni* produces eicosanoids that stimulate vasodilation (e.g., the cyclo-oxygenase products prostaglandin D_2 and prostaglandin E_2), as well as 5-lipoxygenase products that are able to induce vasoconstriction [e.g., leukotriene C_4 and 15-hydroxyeicosatetraenoic acid (15-HETE)] [76]. It has been shown that schistosome vasoconstrictors are released in smaller amounts than the vasodilators [76,77]. This suggests that eicosanoids play a role in the stimulation of vasodilation during infection. However, this difference in production of vasoconstriction-promoting and vasodilation-promoting eicosanoids could also merely reflect differences in biological activity, and therefore, the true impact of schistosome eicosanoids on the vascular tone during infection remains unclear.

Another potential mechanism to induce vasodilation by schistosomes is through the actions of the schistosome proteins sK1 and SmSP1 [46,47]. sK1 is a schistosome protein with kallikrein-like activity, and SmSP1 has homology to mouse kallikrein, but kallikrein-like activity has not been described for SmSP1 [46,47]. As discussed before, *S. mansoni* sK1 and, potentially, SmSP1 convert kininogen to the potent vasodilator bradykinin [46].

Concluding remarks

According to Virchow's triad, schistosomes are expected to be potent activators of blood coagulation. However, the parasite has evolved several mechanisms to actively inhibit blood coagulation and manipulate the vascular tone, ensuring long-term survival in the host's bloodstream (Table 1). Many experiments showed that primary and secondary haemostasis can be inhibited by the parasite through a combination of schistosome-specific mechanisms that inhibit key steps in the coagulation cascade and by schistosome proteins that exploit host mechanisms for regulation of haemostasis. Furthermore, schistosome proteins may actively stimulate fibrinolysis and manipulate vascular tone. The many strategies used by schistosomes to interfere with the haemostatic system of their host reflect the complex host–parasite relationship. Although some major discoveries in manipulation of blood coagulation by schistosomes have been made, additional studies are required to further understand these mechanisms and determine their importance during infection. This would provide more insight into pathways involved in maintaining the parasitic life cycle within the host and may reveal new targets for the development of anti-schistosome drugs or vaccines. Furthermore, mechanisms described here might also be applicable to other blood-dwelling pathogens and may reveal broad targets for the development of drugs to be used against these pathogens. In addition, schistosome molecules that interfere with the haemostatic system could form potential new antithrombotic or thrombolytic drugs for the treatment of haemostatic disorders.

Table 1. Proposed interference with the haemostatic system and vascular tone by schistosome molecules.

Target	Schistosome molecule	Effect
Hypocoagulation mechanisms		
Primary haemostasis		
ADP	SmAP [40,41]	Hydrolysis of ADP to AMP and adenosine, a competitive inhibitor of platelet aggregation and degranulation.
	SmATPDase1 [44,45]	Hydrolysis of ATP and ADP to AMP.
	SmPDE [43]	Hydrolysis of ATP and AMP.
HMWK	sK1 [46]	Conversion of HMWK to bradykinin. Bradykinin stimulates the release of PGI_2, an inhibitor of platelet degranulation, from endothelial cells.
	SmSP1 [47]	Homology to mouse plasma kallikrein.
Secondary haemostasis		
Factor XII	Schistosome homogenate [55-57]	Inhibition of the conversion of coagulation factor XII to XIIa and the actions of XIIa.
Thrombin	Sm22.6 [58]	Inhibitor of thrombin activity.
Antithrombin	Heparin-like molecules [63]	Potential increase of the activity of antithrombin.
	SHW 4-2 [65]	Sequence similarity to antithrombin.
Hyperfibrinolytic mechanisms		
Plasminogen	Enolase [68,69,72]	Binding and conversion of plasminogen to plasmin by t-PA.
	GAPDH [68]	Binding of plasminogen.
	Annexin [70]	Binding and conversion of plasminogen to plasmin by t-PA.
Vascular tone		
HMWK	sK1 [46]	Conversion of HMWK to bradykinin.
	SmSP1 [47]	Homology to mouse plasma kallikrein.
Vasodilation	Eicosanoids [20]	Both vasodilating and vasoconstricting effects.

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3

CHAPTER 3

Hemostatic changes in urogenital schistosomiasis haematobium: a case-control study in Gabonese schoolchildren

Mirjam M. Mebius[1,2], Ayola A. Adegnika[3,4,5], Jeannot F. Zinsou[3,4], Jean Claude Dejon Agobe[3,4], Josiane Y. Honkpehedji[3,4], Maria Yazdanbakhsh[5], Govert J. van Dam[5], Paul L. A. M. Corstjens[6], Aloysius G. M. Tielens[1,7], Philip G. de Groot[2], Rolf T. Urbanus[2], Jaap J. van Hellemond[1]

[1] Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam, the Netherlands
[2] Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[3] Centre de Recherches Médicales de Lambaréné (CERMEL), BP: 118 Lambaréné, Gabon
[4] Institut für Tropenmedizin, Universität Tübingen, Tübingen, Germany
[5] Department of Parasitology, Leiden University Medical Center, Leiden, the Netherlands
[6] Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, the Netherlands
[7] Department of Biochemistry and Cell Biology, Faculty Veterinary Medicine, Utrecht University, the Netherlands

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Abstract

In many tropical areas schistosomiasis is a major health problem causing hepatosplenic, intestinal, or urogenital complaints. Hepatosplenic schistosomiasis mansoni is also characterized by blood coagulation abnormalities. Liver pathology plays a role in development of hemostatic changes and the parasitic infection may directly affect coagulation. However, these contributing factors cannot be studied separately in hepatosplenic schistosomiasis infections.

This pilot study provides insight in hemostatic changes in urinary schistosomiasis by studying coagulation parameters in schistosomiasis haematobium infected Gabonese schoolchildren. Selection on urinary schistosomiasis patients without hepatosplenic complaints allows investigation of direct effects of the parasite on hemostasis. Levels of von Willebrand Factor (VWF) antigen, active VWF, and osteoprotegerin were elevated, indicating inflammation-mediated endothelial activation. In contrast to hepatosplenic schistosomiasis, thrombin-antithrombin complex and D-dimer levels were not affected. Despite its small sample size, this study clearly indicates that *S. haematobium* directly alters the activation status of the endothelium, without initiation of coagulation.

Introduction

The tropical disease schistosomiasis is caused by blood-dwelling parasites of the *Schistosoma* genus. The major disease causing species in humans are *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum* [1]. With almost 800 million people at risk of infection, schistosomiasis is the second major parasitic disease after malaria [2].

The various species of *Schistosoma* reside in blood vessels at different locations in the body and these specific locations determine pathology. *Schistosoma mansoni* and *Schistosoma japonicum* reside in the mesenteric plexus and chronic infection results in the development of intestinal or hepatosplenic schistosomiasis, affecting intestine, liver, and spleen [1]. In contrast, *Schistosoma haematobium* resides in the perivesicular plexus. Infection with this species results in urogenital schistosomiasis, affecting mainly the urogenital tract although hepatic disease may occur in severe infections [1,3].

In addition to intestinal and hepatic disease, the blood coagulation system is affected in schistosomiasis mansoni patients [4]. These individuals are prone to bleeding as a result of coagulation factor deficiencies, and often have reduced platelet counts (reviewed by Tanabe [5]). Several factors are presumed to contribute to the loss of coagulation factors and platelets. First, consumption of coagulation factors through continuous activation of coagulation may be involved, since markers of active coagulation, such as thrombin-antithrombin complexes (TAT), and markers of fibrinolysis, such as D-dimers, are both elevated in these patients. This activation of coagulation can be induced by the parasite itself, but it is also assumed that immune responses to schistosomes play a role in the development of hemostatic abnormalities in hepatosplenic schistosomiasis mansoni, for example through stimulation of monocyte tissue factor expression [6]. Second, the majority of coagulation factors are synthesized by the liver. Therefore, impaired liver function, resulting in reduced hepatic synthesis or clearance of activated coagulation factors, could contribute to the bleeding phenotype. It remains challenging to unravel the contribution of the parasite and liver damage to the development of hemostatic abnormalities in hepatosplenic schistosomiasis mansoni patients, since these factors cannot be separated in hepatosplenic disease.

This pilot study aims to elucidate the contribution of the parasite to the development of coagulation abnormalities in *Schistosoma* infections by studying hemostatic parameters in schoolchildren infected with *S. haematobium*. Since *S. haematobium* infections primarily cause urogenital schistosomiasis, strict selection on *S. haematobium* infected individuals without hepatosplenomegaly limits the influence of impaired hepatic function and allows investigation of the effect of only the parasitic infection on the development of coagulation abnormalities.

Materials and methods

Participants and sample collection

Ten schoolchildren with non-hepatosplenic schistosomiaisis haematobium and four healthy controls without *S. haematobium* infection were recruited at the Centre de Recherches Médicales de Lambaréné (CERMEL), Lambaréné, Gabon. The study was approved by the CERMEL Institutional Ethics Committee (CEI, CEI-MRU 002 26/1/2016). Participants were schoolchildren without hepatosplenic complaints from Zilé-PK15 areas, *S. haematobium* endemic areas surrounding Lambaréné [7]. Informed consent was obtained from parents or legal guardians of all children and all infected individuals received treatment with praziquantel after sample collection. Venous blood was collected in a tube containing 1:10 volume of anticoagulant 3.2% sodium citrate solution. Whole blood was analyzed with the Pentra 60 analyzer (Horiba, Kyoto, Japan). Citrated platelet poor plasma was obtained by two-fold centrifugation of citrated blood at 2000xg for 15 minutes. Citrated plasma was aliquoted and stored at -80˚C.

Determination of *S. haematobium* infection

Circulating anodic antigen (CAA) levels were determined in 20 µL citrated plasma with the wet reagent SCAA20 (standard serum/plasma circulating anodic antigen test on 20 µL TCA extract) lateral flow assay as described by Corstjens *et al.* [8]. Samples were treated with 4% trichloroacetic acid (TCA) to remove interfering components. CAA levels were calculated with a four parametric curve fitting method, using a standard dilution curve of the TCA-soluble fraction of schistosome adult worm antigen (AWA-TCA), which contains approximately 3% (w/w) CAA. The lower detection limit of the SCAA20 was 150 pg/mL AWA-TCA (= 5 pg CAA/mL). Participants were considered positive when CAA levels were above 5 pg/mL. In addition, for all participants a midstream urine sample was collected during the daytime and at least 10 mL urine was passed through a 12.0 µm polyamide N filter (Millipore, City, State, Country). *S. haematobium* eggs were detected by microscopic examination.

Enzyme-linked immunosorbent assay

Levels of D-dimer were measured in plasma with the Technozym D-dimer ELISA kit (Stago BNL, Leiden, The Netherlands) according to manufacturer's instructions. Levels of von Willebrand Factor (VWF) antigen (VWF:ag), active VWF, ADAMTS-13 antigen (ADAMTS-13:ag), osteoprotegerin (OPG), and thrombin-antithrombin (TAT) complexes were measured in plasma with ELISA. 96-well or 384-well white maxisorp microtiter plates (Thermo Scientific, Roskilde, Denmark) were coated overnight at 4˚C with polyclonal rabbit-anti-human VWF (0.775 µg/mL; A0082; DAKO, Carpinteria, California, U.S.A.), AU/VWFa-11 nanobody [9] (1.98 µg/mL; LKCH), D053 (0.5 µg/mL;

Sanquin, Amsterdam, The Netherlands), mouse-anti-human OPG/TNFRSF11B (1 µg/mL; R&D Systems, Minneapolis, Minnesota, U.S.A.), or sheep-anti-human Thrombin (1 µg/mL; Stago BNL, Leiden, The Netherlands) in coating buffer (40mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6). Plates were washed four times with washing buffer (phosphate buffered saline (PBS (ThermoFisher Scientific, Waltham, Massachusetts, U.S.A.), 0.05% Tween20, pH 7.4) between all incubation steps and blocked for one hour at room temperature with blocking buffer (PBS, 1% BSA, pH 7.4). Citrated plasma and standard were diluted in blocking buffer: 125-fold for VWF:ag ELISA, 5- and 10-fold for active VWF ELISA, 40-fold for ADAMTS-13:ag ELISA, and 4-fold for OPG and TAT ELISAs and incubated for one hour at room temperature. Used standards were serial dilutions of normal pooled plasma (Sanquin, Amsterdam, The Netherlands) with a known concentration of VWF:ag for VWF:ag and ADAMTS-13:ag, normal pooled serum with known concentrations of TAT and OPG for TAT and OPG ELISAs and VWF depleted plasma (Affinity Biologicals, Ancaster, Canada) supplemented with recombinant R1341Q-VWF for active VWF ELISA. Following sample incubation the plates were incubated at room temperature for one hour with the following detection and secondary antibodies diluted in blocking buffer: peroxidase-conjugated rabbit-anti-human VWF (0.325 µg/mL; DAKO, Carpinteria, California, U.S.A.), peroxidase-conjugated rabbit-anti-human VWF (1.2 µg/mL; DAKO, Carpinteria, California, U.S.A.), biotinylated polyclonal sheep-anti-human ADAMTS-13 (0.25 µg/mL; R&D Systems, Minneapolis, Minnesota, U.S.A.) and streptavidin-mono-HRP (0.83 µg/mL; DAKO, Carpinteria, California, U.S.A.), biotinylated goat-anti-human OPG/TNFRSF11B (100 ng/mL; R&D Systems, Minneapolis, Minnesota, U.S.A.) and streptavidin-mono-HRP (0.25 µg/mL; DAKO, Carpinteria, California, U.S.A.), and peroxidase-conjugated sheep-anti-human antithrombin (0.5 µg/mL; Stago BNL, Leiden, The Netherlands). ELISAs were developed with SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, Massachusetts, U.S.A.) and luminescence was detected with the Synergy2 ELISA reader (BioTek, Winooski, Vermont, U.S.A.). Antigen concentrations were calculated with a five parametric curve fitting method, using a dilution curve of the standard. The lower detection limits of the ELISAs were: 6.8 ng/mL (VWF:ag), 16ng/mL (active VWF), 1.6% (ADAMTS-13:ag), 10.6 pg/mL (OPG), and 2.8 pM (TAT).

Ristocetin co-factor activity assay

Citrated plasma was diluted 7-fold in imidazole buffer (100 mM imidazole, 100 mM NaCl, pH 7.4). A standard dilution curve was prepared of diluted normal pooled plasma (Sanquin, Amsterdam, The Netherlands). To 70 µl sample 200 µl BC von Willebrand Reagent (Siemens healthcare, Erlangen, Germany) was added in a 96-well clear maxisorp microtiter plate (Thermo Scientific, Roskilde, Denmark). Following 10 minutes incubation at 37˚C and 20 minutes incubation at room temperature to allow platelet aggregates to precipitate, 100 µl of the mixture was transferred to a new

plate and non-aggregated platelets were measured at 350 nm. Ristocetin co-factor activity (VWF:RCo) was calculated with a five parametric curve fitting method, using a dilution curve of the standard. The lower detection limit of the assay was 3% VWF:RCo.

ADAMTS-13 activity measurement

ADAMTS-13 activity was measured in a kinetic assay using the fluorescence resonance energy transfer substrate VWF73 (FRETS-VWF73; Peptide institute, Osaka, Japan) as previously described [10]. Samples were 10-fold diluted in Tris buffer (5 mM Tris, 25 mM $CaCl_2$, 0.005% Tween20, pH 6.0). FRETS-VWF73 was added to a final concentration of 2 μM and fluorescence (λ_{ex} 340 nm, λ_{em} 450 nm) was measured in a 96-well black microtiter plate (Thermo Scientific, Roskilde, Denmark) every 30 seconds for one hour at 30˚C with a Spectramax M2e device (Molecular devices, Sunnyvale, U.S.A.).

Statistical analysis

Analyses were done using PRISM software (GraphPad, San Diego, California, U.S.A., version 6.01). Infected and non-infected control groups were compared using Mann-Whitney test and $P \leq 0.05$ was considered statistical significant.

Results and discussion

We included ten schoolchildren with schistosomiasis haematobium and four healthy children from *S. haematobium* endemic, rural areas surrounding Lambaréné (Gabon) (Table 1). Infection status was based on circulating anodic antigen (CAA) levels in plasma [8] combined with detection of viable eggs in urine, since detection of eggs in urine as only method to confirm infection (gold standard) has a low sensitivity [1]. Hepatomegaly was absent in all studied individuals and splenomegaly was absent in nine out of ten infected and in all non-infected individuals, therefore, all infected children were characterized as strictly urogenital schistosomiasis haematobium.

In hepatosplenic schistosomiasis mansoni levels of von Willebrand Factor (VWF) antigen (VWF:ag) are highly elevated and inversely correlate with platelet counts, as patients experience thrombocytopenia [11]. To determine the effect of *Schistosoma* infection on VWF levels in urinary schistosomiasis, we determined VWF:ag, active VWF, and von Willebrand Factor ristocetin cofactor activity (VWF:RCo) in our study participants. VWF is secreted by endothelial cells and circulates in a globular conformation in which the platelet binding A1-domain is inaccessible [9]. Under blood flow VWF unfolds and adopts an active platelet-binding conformation (active VWF) that can be detected using the AU/VWFa-11 nanobody [9]. Besides flow, ristocetin can also be used to unfold VWF and study its binding to platelets (VWF:RCo). Levels of

Table 1. Participant characteristics.

	***S. haematobium* infected children**	**Controls**
Number, n	10	4
Age (y), mean (SD)	11 (3)	8 (1)
Male, n (%)	5/10 (50)	3/4 (75)
Weight (kg), mean (SD)	37 (13)	27 (2)
Height (m), mean (SD)	1.47 (0.16)	1.34 (0.03)
CAA (pg/mL), mean (SD)	2166 (3062)	0 (0)
Egg count positive, n (%)	9/10 (90)	0/4 (0)
Egg count/10 mL urine, mean (SD)	272 (289)	0 (0)
Parasitic co-infections, n (%)		
Ascaris	0/8 (0)	0/3 (0)
Ancylostoma	0/8 (0)	0/3 (0)
Trichuris	3/8 (38)	1/3 (33)
Missing	2	1
Whole blood analysis, mean (SD)		
Hemoglobin (g/dL)	11.0 (1.9)	11.7 (1.3)
Missing	1	0
Platelets (n x 10^9/L)	253 (66)	322 (54)
Missing	1	0
White blood cells (n x 10^9/L)	10.5 (3.5)	10.1 (2.9)
Missing	1	0
Eosinophils (n x 10^9/L)	1.8 (0.8)	1.9 (1.3)
Missing	5	1
Lymphocytes (n x 10^9/L)	4.2 (0.9)	3.6 (0.5)
Missing	1	0
Monocytes (n x 10^9/L)	0.7 (0.2)	0.9 (0.2)
Missing	1	0
Splenomegaly, n (%)	1/9 (11)	0/4 (0)
Missing	1	0
Hepatomegaly, n (%)	0/9 (0)	0/4 (0)
Missing	1	0

Data are number with percentage or mean with SD. CAA indicates circulating anodic antigen.

VWF:ag and active VWF were significantly increased in infected children compared to non-infected controls (Fig. 1a and b, $P = 0.002$ and $P = 0.004$, respectively, dotted lines indicate normal range). The elevated VWF:ag levels indicate either increased secretion or reduced breakdown of VWF. The proportion of VWF that is active (percentage active VWF) is slightly decreased in infected children compared to non-infected individuals, indicating that increased conformational activation of circulating VWF is not responsible for the observed increase in active VWF (Fig. 1c, $P = 0.024$). In contrast to increased VWF:ag and active VWF levels, VWF:RCo was low or absent in all individuals (Fig. 1d, $P = 0.6234$, dotted lines indicate normal range). As all individuals had normal platelet counts ((253 ± 66) x 10^9/L and (322 ± 54) x 10^9/L in infected and non-infected individuals, respectively ($P = 0.1063$)) and plasma from Dutch controls showed normal VWF:RCo activity (data not shown), this observation cannot be explained by low platelet counts or poor performance of the VWF:RCo assay. Reduced or absent platelet aggregation on ristocetin in Africans compared to Europeans has been reported previously [12,13]. Reduced platelet function was attributed to a plasma inhibitor of ristocetin-induced platelet aggregation [12,13], though genetic polymorphisms in VWF could also affect the ristocetin-based activity assay [14]. It is, however, unclear whether these accounted for the observed low VWF:RCo activity in our study. In summary, in urinary schistosomiasis haematobium VWF:ag and active VWF levels are elevated and do not correlate with platelet counts as thrombocytopenia is absent.

Changes in levels of VWF:ag and active VWF could reflect defects in the VWF-degrading protease ADAMTS-13 [9], but could also indicate endothelial activation [15]. To study functionality of ADAMTS-13, both ADAMTS-13:ag levels and ADAMTS-13 activity were determined. ADAMTS-13:ag levels and ADAMTS-13 activity were normal in both infected and non-infected children (Fig. 1e and f, $P = 0.4116$ and $P = 0.5774$, respectively), so no defects in VWF-degradation are present.

Next, endothelial activation was determined using osteoprotegerin (OPG) levels as marker for inflammation-mediated endothelial activation [16]. Elevated levels of OPG were observed in the *S. haematobium* infected children versus non-infected controls (Fig. 2g, $P = 0.036$, dotted line indicates cut-off value). Simultaneous elevation of both VWF:ag and OPG during schistosomiasis haematobium indicates inflammation-mediated endothelial activation and is in line with the reported co-localization of these markers in endothelial Weibel Palade bodies and their simultaneous secretion upon endothelial cell stimulation [16].

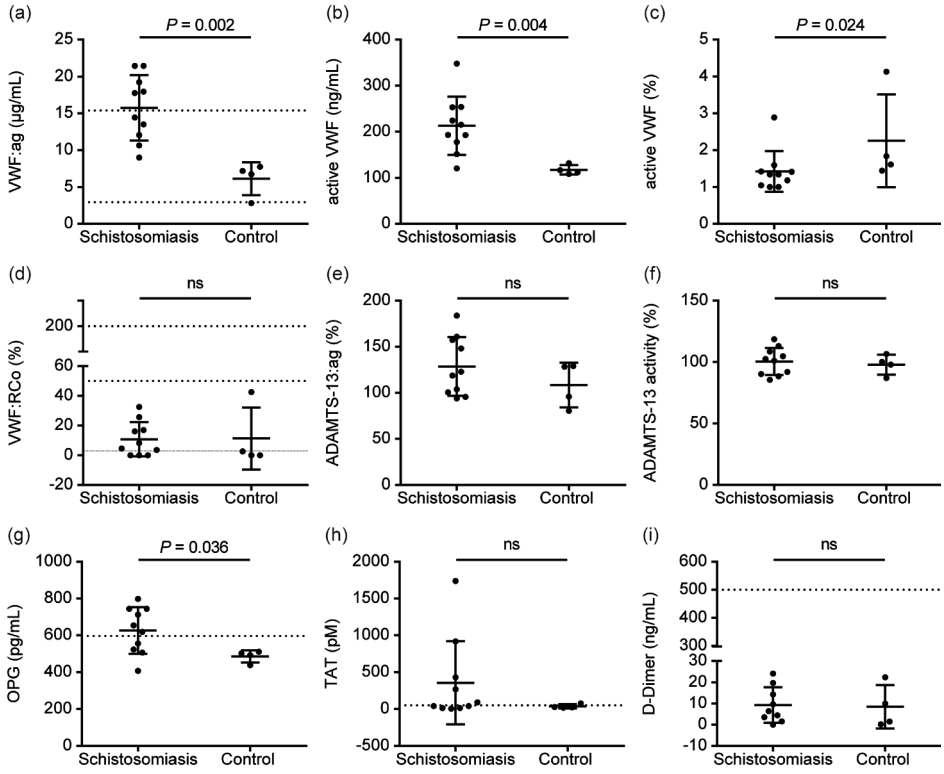

Figure 1. Hemostatic parameters in *S. haematobium* infected schoolchildren.

VWF:ag levels (a), active VWF levels (b), ADAMTS-13:ag levels (e), OPG levels (g), TAT levels (h), and D-dimer levels (i) were measured with ELISA in citrated plasma of *S. haematobium* infected schoolchildren and non-infected controls. Percentage of active VWF (c) was calculated with the determined active VWF and VWF:ag levels. VWF:RCo (d) was determined with the BC von Willebrand Reagent. ADAMTS-13 activity (f) was determined with the FRETS-VWF73 assay. Dotted lines indicate normal ranges (a, d) or cut-off values (g, h, i). Statistical analysis was performed with a Mann-Whitney test. $P \leq 0.05$ was considered statistical significant (ns = not significant).

Several mechanisms may explain activation of the vessel wall. Secretion of OPG is stimulated by the inflammatory cytokines TNF-α and IL-1β [16]. In hepatosplenic schistosomiasis mansoni patients TNF-α production by PBMC is elevated and plasma levels of soluble TNF receptor I and II are increased [17], suggesting contribution of inflammatory cytokines produced during infection to the observed increased OPG levels. However, no correlation between serum levels of TNF-α and infection status or disease severity is observed in schistosomiasis haematobium [18]. Passage of schistosome eggs through the vessel wall or egg-derived materials could also induce endothelial activation [19]. Furthermore, endothelial adhesion of adult *S. mansoni* through their ventral sucker could damage the endothelium [20] and the presence of the adult worm pair in the small veins could alter blood flow, leading to endothelial activation [4].

The observation that endothelial activation occurs during urinary schistosomiasis is of importance as elevated levels of VWF, OPG, and endothelial activation are associated with thrombosis and cardiovascular disease [21,22]. Ongoing coagulation has been demonstrated in hepatosplenic schistosomiasis mansoni, reflected by increased levels of TAT and D-dimer [5]. We therefore investigated whether the observed endothelial activation in urinary schistosomiasis haematobium results in a procoagulant phenotype in urinary schistosomiasis. In contrast to hepatosplenic schistosomiasis mansoni patients, TAT and D-dimer levels were generally low in our study population and we could not demonstrate differences in urinary schistosomiasis patients compared to the non-infected individuals (Fig. 2h and i, $P = 0.4116$ and $P = 0.7524$, respectively, dotted line indicates cut-off value), indicating that there is no ongoing coagulation or fibrinolysis in these individuals. However, five of the infected children had TAT levels above the normal threshold, without elevated D-dimer levels. Coagulation activation may have occurred in these individuals leading to low-level thrombin activation, followed by subsequent inhibition of the formed thrombin by antithrombin, leading to the formation of TAT without fibrin formation, which explains the absence of D-dimers in these individuals.

We here report the first study on direct effects of *S. haematobium* on hemostatic abnormalities in urinary schistosomiasis haematobium patients without hepatosplenic complaints. Despite the small sample size of this pilot study, our observations indicate that *S. haematobium* directly alters the activation status of the vessel wall. This is not unexpected as the relatively large size of the adult worm pairs in the blood vessel will provoke substantial turbulence and increased shear stress along the vessel wall, processes known to induce endothelial activation [4]. In contrast to reports on coagulation abnormalities in hepatosplenic schistosomiasis mansoni, ongoing coagulation and fibrinolysis, which is reflected by elevated levels of TAT and D-dimer, was absent in urinary schistosomiasis patients. In schistosomiasis

mansoni the reported coagulopathy may be aggravated as a result of impaired liver function [5]. The direct effects of the parasite on the vessel wall are also of interest, as endothelial activation and elevated levels of VWF are linked to thrombosis. We therefore hypothesize that direct activation of the endothelium by *S. haematobium* may be involved in initiation of coagulation abnormalities in schistosomiasis. Interestingly, a VWF-cleaving peptidase was recently discovered in *S. mansoni*, which suggests that schistosomes have strategies to counteract endothelial activation and elevated levels of VWF, which are unfavorable for both the parasite and its host, as this can lead to thrombosis (Mebius *et al.*, manuscript in preparation). Larger studies on endothelial activation in schistosomiasis haematobium patients with various disease severities are required to confirm our observations and elucidate mechanisms involved in schistosomal-induced endothelial activation and consequences of this endothelial activation on hemostasis.

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4

CHAPTER 4

Cleavage of von Willebrand Factor by a surface protease (SmCB2) of the flatworm pathogen, *Schistosoma mansoni*

Mirjam M. Mebius[1,2], J. Mirjam A. Damen[3], Simone Haeberlein[4], Renske A. van Gestel[3], James H. McKerrow[5], Conor R. Caffrey[5], Christoph G. Grevelding[4], Aloysius G. M. Tielens[1,6], Philip G. de Groot[2,7], Rolf T. Urbanus[2], Jaap J. van Hellemond[1]

[1] Department of Medical Microbiology and Infectious Diseases, Erasmus MC University Medical Center, Rotterdam, Rotterdam, the Netherlands
[2] Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands
[3] Biomolecular Mass Spectrometry & Proteomics, Utrecht Institute for Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, the Netherlands
[4] Institute of Parasitology, BFS, Justus-Liebig-University Giessen, Giessen, Germany
[5] Center for Discovery and Innovation in Parasitic Diseases, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA 92093, USA
[6] Department of Biochemistry and Cell Biology, Faculty Veterinary Medicine, Utrecht University, the Netherlands
[7] Present address: Synapse Research Institute, Maastricht, the Netherlands

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Abstract

Background and objective

In schistosomiasis, adult *Schistosoma* worms reside for prolonged periods in the vasculature of their human host. The size of the worm pair and the interaction with endothelium is presumed to activate coagulation. As a result of endothelial activation, plasma levels of von Willebrand Factor (VWF) are increased in schistosomiasis, which could result in hemostatic activation. The adult worms could therefore form a potential prothrombotic threat. However, no increased thrombosis is observed in schistosomiasis patients.

VWF is a plasma protein involved in aggregation of platelets and plays an important role in the hemostatic process. Functionality of VWF is controlled through proteolysis by the metallopeptidase ADAMTS13 and deficiency of ADAMTS13 is linked to a severe form of thrombotic microangiopathy called thrombotic thrombocytopenic purpura (TTP).

Methods

We examined whether *Schistosoma mansoni* contains a peptidase that can cleave human VWF and determined its characteristics.

Results and conclusion

We discovered that *S. mansoni* possesses a proteolytic enzyme that acts on multimeric VWF. This *S. mansoni* VWF-cleaving peptidase belongs to the class of divalent cation-independent cysteine peptidases and turned out to be SmCB2, a well-studied tegumental cathepsin B. Plasma components from healthy donors partially inhibit the VWF-cleaving peptidase, which is however not sensitive to plasma components specific for TTP patients. The discovery of SmCB2 as a VWF-cleaving peptidase provides more insight into the mechanisms used by schistosomes to limit coagulation activation and forms a possibility for new therapeutic strategies for disorders in which control of VWF function is disturbed, such as TTP.

Introduction

Parasitic flat worms of the *Schistosoma* genus are blood-dwelling worms that are the causative agent of the tropical disease schistosomiasis. On average, the adult worms reside in the human mesenteric (*Schistosoma* mansoni and *S. japonicum*) or perivesicular veins (*S. haematobium*) for three to five years, but their individual lifespan can be as long as 30 years [1,2]. The prolonged presence of adult schistosome pairs in the veins of their human host poses a potential prothrombotic threat [3–6]. Attachment of the parasite to the vessel wall and the production of inflammatory cytokines result in endothelial activation, leading to an increased hemostatic response. Additionally, disturbances in blood flow due to the large size of the parasite and prothrombotic molecules on the surface of the worms could trigger coagulation. Besides direct activation of the hemostatic system by the adult parasite, the eggs that are produced by the worm pair are potent, direct activators of platelets [7]. The eggshell directly binds von Willebrand Factor (VWF), which is essential for adhesion of platelets to the vessel wall during normal hemostasis [8]. Also, extravasation of the eggs through the vessel wall will induce endothelial damage or endothelial activation, leading to coagulation [9]. A study in hepatosplenic schistosomiasis patients showed that VWF levels are increased during infection [10] and in a small study of urinary schistosomiasis haematobium patients we observed that this increase in VWF levels is caused by inflammation mediated endothelial activation (Mebius et al., Chapter 3 of this thesis).

VWF is a plasma glycoprotein that mediates adhesion and aggregation of platelets. The protein is synthesized by endothelial cells and megakaryocytes as long multimers with a molecular weight of up to more than 20,000 kDa [11]. The majority of circulating VWF is synthesized by endothelial cells [12]. Endothelial cells secrete part of the produced VWF via the constitutive pathway, but a substantial part is stored in Weibel-Palade bodies, which is released upon stimulation of the endothelium with for example the inflammatory cytokines IL-8 and TNF-α [13,14].

Since we observed that inflammation-mediated endothelial activation occurs during schistosome infection and plasma VWF levels are increased without apparent activation of coagulation, we hypothesised that schistosomes counteract the increased VWF secretion. In particular we were interested to test whether VWF could be proteolytically modified by adult *S. mansoni* to counter the potential prothrombotic effect of increased VWF secretion during schistosome infection.

Most of the secreted VWF consists of ultra-large VWF (ULVWF) multimers that are highly prothrombotic and are formed by disulfide linkage of VWF monomers during synthesis [11]. Prothrombotic activity of VWF is regulated during normal hemostasis through limited cleavage by the metalloproteinase ADAMTS13 (A Disintegrin And

Metalloproteinase with a ThromboSpondin type 1 motif, member 13) [11]. ULVWF multimers will adopt a globular conformation in plasma [15]. High shear rates upon passage through the microvasculature result in (partial) unravelling of globular ULVWF, exposing the cryptic binding and cleavage sites for ADAMTS13, resulting in the cleavage of VWF by ADAMTS13 within its A2 domain [15]. This results in the typical multimeric pattern of VWF triplets observed after gel electrophoresis, where the major VWF band is flanked by faster and slower migrating satellite bands, which appear upon cleavage [11]. ADAMTS13 deficiency is associated with thrombotic thrombocytopenic purpura (TTP), a rare but severe form of thrombotic microangiopathy caused by aggregation of platelets by ULVWF [15,16]. In addition to ADAMTS13, several other peptidases have been described to cleave VWF [17–22], providing alternative routes for control of VWF function. These peptidases include the hemostatic-fibrinolytic proteins thrombin and plasmin [17,18], calpain [21,22], and the leukocyte-derived proteinase 3, neutrophil elastase, matrix metalloproteinase (MMP)-8, MMP-9, and cathepsin G [18,19], all of which could be involved in local control of VWF function restricted to thrombi. Additionally, VWF cleavage by pathogens has been described during infection with Bacillus anthracis by the bacterial metallopeptidase neutral protease 599 (Npr599) and immune inhibitor A metallopeptidase (InhA), resulting in proteolytically cleaved VWF that is deficient in collagen binding and platelet aggregation activity [20]. Furthermore, kaouthiagin, a metalloproteinase from snake venom has also been described to bind and cleave VWF, resulting in loss of ristocetin-induced platelet aggregation and the collagen-binding activity of VWF [23].

Schistosomes produce a broad range of peptidases that are vital at many stages of infection [24]. Schistosomal peptidases facilitate host invasion, transformation of cercariae to schistosomula, acquisition of nutrients by digestion of host protein, reproduction, and immunomodulation [24–27]. Interference with peptidase activities of schistosomes through the use of peptidase inhibitors is a successful therapeutic approach [28], and cysteine peptidases appear to be good candidates for vaccine development against *S. mansoni* [29]. Although many peptidases of *S. mansoni* have been extensively studied, to date none of these peptidases have been described to interfere with the functionality of VWF. This study is the first to evaluate schistosome preparations for proteolytic activity towards VWF, which will shed light on the interaction between *S. mansoni* and VWF during infection.

Materials and methods

Plasma samples

Pooled platelet-poor citrated plasma was obtained from blood of 50 healthy volunteers (after informed consent) and was prepared by centrifugation (twice 10 min, 2000x g) (Sanquin, Amsterdam, the Netherlands). Blood samples from TTP patients were obtained with informed consent from TTP patients present at the annual TTP patient day (Sanquin 2016, Amsterdam, the Netherlands) and platelet-poor citrated plasma was prepared by centrifugation (twice 10 min, 2000x g). Plasma samples were stored at -80 °C.

Parasites

Intact adult *S. mansoni* pairs were isolated from Golden hamsters seven weeks after infection with 600 cercariae per hamster. Hamsters were anesthetized with isoflurane and perfused with 0.9% NaCl solution. Isolated worms were rinsed in 0.9% NaCl and frozen in 0.9% NaCl for storage at -20 °C. Worm homogenate (WH) was prepared in HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, pH 7.4) with a Teflon potter, after which nuclei and unlysed cells were removed by centrifugation at 500x g for 5 minutes at 4°C. Soluble worm fraction was prepared by subsequent centrifugation of the WH at 14000x g for 30 minutes at 4°C. Protein concentration in the schistosomal samples were determined according to the method of Lowry *et al.* [30].

FRETS-VWF73 substrate assay

Cleavage of the FRETS-VWF73 substrate (AnaSpec, Fremont, Ca, USA) was measured as published with some modifications [31]. FRETS-VWF73 substrate was dissolved at a concentration of 100 µM in 25% (v/v) dimethyl sulfoxide (DMSO) and diluted to 4 µM in assay buffer (5 mM Bis-Tris, 25 mM CaCl2, 0.005% Tween-20, pH 6.0). Plasma samples or schistosomal fractions, where indicated supplemented with peptidase inhibitors, were diluted in assay buffer. Peptidase inhibitors used were N-ethylmaleimide (NEM; 10 mM), leupeptin (Leu; 100 µM), pepstatin A (PepA; 5 µM), phenylmethylsulfonyl fluoride (PMSF; 1 mM), ethylenediaminetetraacetic acid (EDTA; 5 mM), and L-cysteine (L-cys; 50 mM). Recombinant expressed SmCB2 (rSmCB2) was a kind gift from Dr. Conor R. Caffrey (Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, USA) and was diluted and activated for 5 minutes at room temperature in citrate buffer (100 mM trisodium citrate, 2 mM DTT, pH 5.5) and, where indicated, supplemented with 5 mM EDTA. FRETS-VWF73 substrate (2 µM) in assay buffer was added to initiate the assay. Fluorescence was monitored for 1 hour at 30°C on a Spectromax M2 fluorescence microplate reader (MTX Lab Systems, Bradenton, FL, USA) equipped with a 340 nm excitation filter and 450 nm emission filter. Initial rates of increase in fluorescence are represented as mean ± SD.

Cleavage of native VWF

Purified VWF (Biotest AG, Dreieich, Germany, 227 µg/mL) in HBS was incubated with 1 mg/mL WH and/or 3.6% platelet-poor citrated plasma in 1.5 M urea buffer (1.5M urea, 2.5 mM Tris, pH = 8) containing 4.5 mM BaCl in a final volume of 110 µL at 37°C for 2, 4, or 8 hours. Reactions were terminated by addition of 4 volumes (440 µL) of denaturing buffer (9M Urea, 0.1% bromophenol blue, 2 mM ethylenediaminetetraacetic acid (EDTA), 35 mM Tris, 1% SDS, pH 6.7) and incubation for 30 minutes at 56°C.

Samples were separated by gel electrophoresis on a stacking gel of 1% HGT agarose (Lonza, Basel, Switzerland) in stacking gel buffer (4 mM EDTA, 70 mM Tris, 0.4% SDS, pH 6.7) and a running gel of 2.5% HGT agarose in running gel buffer (100 mM Glycine, 200 mM Tris, 0.4% SDS, pH 9.0) on a SE 600 Ruby gel electrophoresis unit (Amersham Biosciences, Little Chalfont, UK) for 20 to 24 hours at 4°C and constant amperage of 40 mA. Used gel electrophoresis running buffers were upper chamber buffer (150 mM glycine, 100 mM Tris, 0.1% SDS, pH 8.45) and lower chamber buffer (75 mM glycine, 50 mM tris, pH 8.45). Gels were fixed in fixative (1:10:9 acetic acid: isopropanol: water) for 1 hour at room temperature, and subsequently washed 3 times with water. In gel staining was performed by incubation with 1:5,000 diluted polyclonal rabbit-anti-human VWF antibody (A0082, DAKO, Carpinteria, CA, USA), followed by incubation with 1:10,000 diluted goat-anti-rabbit-Alexa680 (A21076, Invitrogen, Carlsbad, CA, USA). Before use, both antibodies were diluted in 1:1 odyssey blocking buffer (LI-COR, Lincoln, NB, USA) and phosphate buffered saline (PBS)-0.1% Tween-20 (137 mM NaCl, 2.7 mM KCl, 9.2 mM Na2HPO4 • H2O, 1.76 mM KH2HPO4, 0.1% Tween-20, pH 7.4). Incubations were performed for 4 hours at room temperature or overnight at 4°C and between the incubations the gels were washed twice with PBS-0.1% Tween-20. Gels were visualized on a near-infrared scanner (Odyssey, LI-COR, Lincoln, NB, USA) with Odyssey V3.0 software (LI-COR, Lincoln, NB, USA).

Anion exchange chromatography fractionation

Anion exchange chromatography fractionation was performed on Q Sepharose fast flow resin (GE Healthcare Life Sciences, Boston, MA, USA) in a Tricorn 10/50 column (bed volume 4 mL, GE Healthcare Life Sciences, Boston, MA, USA). The column was run on an ÄKTA pure system (GE Healthcare Life Sciences, Boston, MA, USA) at 1 mL/min with running buffer (20 mM triethanolamine, pH 8.0). *S. mansoni* soluble worm fraction was prepared in running buffer as described above and 9 mL was loaded onto the column using a 10 mL Superloop (GE Healthcare Life Sciences, Boston, MA, USA).

Fractions (500 µL per fraction) were step-wise eluted with 250 mM, 500 mM, and 1 M NaCl in running buffer and kept at 4 °C. Peptidase activity was tested in 40 µL fraction with the FRETS-VWF73 substrate assay. The three fractions with the highest activity in the FRETS-VWF73 substrate assay were pooled. Anion exchange chromatography was performed in triplicate.

Mass spectrometry

Pooled active fractions and flow-through from anion exchange chromatography (performed in triplicate) were separated by SDS-PAGE. The lanes of the SDS-PAGE were subsequently divided into 4 bands approximately equal in size (only including proteins with molecular weights smaller than ~80 kDa) and proteins were subjected to in-gel tryptic digestion as described by Shevchenko *et al.* [32]. After digestion samples were analyzed on an Orbitrap Q-Exactive (Thermo Fisher Scientific, Waltham, MA, USA) connected to a UHPLC Proxeon Easy-nLC 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were trapped on a double-fritted trap column (Dr. Maisch Reprosil C18, 3 µm, 2 cm × 100 µm (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany)) and separated on an analytical column (Agilent Zorbax SB-C18, 1.8 µm, 40 cm × 75 µm (Agilent, Santa Clara, CA, USA)). Solvent A consisted of 0.1 M acetic acid, solvent B of 0.1 M acetic acid in 80% acetonitrile. Samples were loaded at a pressure of 800 bar with 100% solvent A. Peptides were separated by a 30 min gradient of 10–30% buffer B followed by 30–100% B in 2 min, 100% B for 2.5 min at a flow rate of 150 nL/min. Full scan MS spectra were acquired in the Orbitrap (350-1500 m/z, resolution 35,000, AGC target 3e6, maximum injection time 250 ms). The 20 most intense precursors were selected for HCD fragmentation (isolation window 1.2 Da, resolution 17,500, AGC target 5e4, maximum injection time 120 ms, first m/z 100, NCE 33%, dynamic exclusion 60 s). The results were filtered using Percolator (Käll *et al.* [33] and Spivak *et al.* [34]) to a false discovery rate (FDR) below 1%. We further only accepted peptides with at least six amino acid residues, a Mascot ion score of at least 20, and search engine rank of 1 and at least 2 identified peptides for protein identification. Scores (Mascot ion score) and peptide spectrum matches (PSM; total number of identified peptide spectra matched for the protein) were used to compare the samples. Only proteins that showed a minimum PSM score of 4 and at least a two times enrichment in PSM were considered as enriched proteins.

Analysis of the data was performed with Proteome Discoverer using the *S. mansoni* database (*Schistosoma*_mansoni_v5.2.fa, Wellcome Trust Sanger Institute, Hinxton, UK, accessible from: ftp://ftp.sanger.ac.uk/pub/project/pathogens/*Schistosoma*/mansoni/Archive/S.mansoni/genome/) for annotation of the peptides. Peptidases were identified based upon the presence of a peptidase KEGG domain and cysteine peptidases were further identified by the presence of "GO:0004197" or the term "Cathepsin" or present on AmiGO 2 as "cysteine-type endopeptidase activity" (http://amigo.geneontology.org/amigo/term/GO:0004197).

RNA interference

Adult *S. mansoni* were isolated from Golden hamsters as described above. Two specific small inhibitory RNAs (siRNAs) were designed for *S. mansoni* Cathepsin B2 (Smp_141610.01) with the help of the on-line IDT RNAi Design Tool (https://eu.idtdna.com/Scitools/Applications/RNAi/

RNAi.aspx) and synthesized commercially (RiBoxx, Radebeul, Germany) with the following sense-strand sequences: cathepsin B-siRNA A 5'-ACAUCACUUUCAAUCCCGCCCCC-3', cathepsin B-siRNA B 5'-UAUACAAAUACGAUCAGACCCCC-3'. The siRNAs were delivered to adult male worms by electroporation according to published protocols with some modifications [36]. Briefly, electroporation was performed in 4 mm cuvettes with 10 males each in 100 µL electroporation buffer (120 mM trehalose, 20 mM HEPES, 1 mM myo-inositol, 1 mM KCl, 1 mM MgCl2, 1 mM K2HPO4, 0.4 mM KH2PO4, 1 mM glutathione, at pH 6.9±0.3) containing 2.5 µg siRNA (siRNA A, siRNA B, or siRNA A+B) or no siRNA as a negative control. A square-wave protocol was applied with a single 20 ms impulse at 125 V and at room temperature (Gene Pulser XCellTM, Bio-Rad, Munich, Germany). After electroporation, the worms were transferred to M199 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Newborn Calf Serum (NCS; Sigma-Aldrich, Berlin, Germany), 1% HEPES (Roth, Karlsruhe, Germany; 1 M), and 1% ABAM (GE Healthcare, Darmstadt, Germany; 10,000 units penicillin, 10 mg streptomycin, and 25 mg amphotericin B per mL) for soaking and incubated for 4 days at 37° C and 5% CO2; 48 hours after electroporation the medium was refreshed. SiRNA incubations were performed in triplicate. Five of the ten worms of each replicate were used for knock-down control by qRT-PCR. The remaining 5 incubated worms were transferred into HBS and stored at -20°C. To assess peptidase activity, *S. mansoni* soluble worm fraction was prepared of these 5 worms in 200 µL HBS and each sample was tested in triplicate in the FRETS-VWF73 substrate assay as described above.

Quantitative reverse transcription PCR

Total RNA from RNAi-treated *S. mansoni* males was extracted using PeqGOLD TriFast (Peqlab, Erlangen, Germany) following the manufacturer's protocol. RNA quality and quantity were checked by electropherogram analysis using the BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Synthesis of cDNA was performed using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) including a genomic DNA wipe out step and using 150 ng of total RNA per reaction. Transcripts were quantified for the target gene Cathepsin B2 (Smp_141610.01), as well as Cathepsin S (Smp_139240.1) and another Cathepsin B (Smp_085180) to detect any off-target effects of the siRNA, and using Smp_008900 (annotated as eukaryotic translation initiation factor 4, eukTF) as reference gene [37]. The following primer pairs (Biolegio, Nijmegen, the Netherlands) were used for qRT-PCRs: SmCB141-RTPCR-5' (5'-ATGTAAAACAAATTGCCAACCAGG-3') + SmCB141-RTPCR-3' (5'-TTGGGAAATCGGCATACACTTC-3'), SmCS-RTPCR-5' (5'-CCAGAATACGTTGATTGGAGA-3')+SmCS-RTPCR-3'(5'-TGGAGTAAGAGTTCCTGTCTTGA-3'), SmCB085-RTPCR-5' (5'- ATTGAAGAATGAACATATTGAACCG-3') + SmCB085-RTPCR-3' (5'-ATAGTTTGAATTCGTTTCCCACC-3'),eukTF-RTPCR-5' (5'-GAGCTTATATGTGAAGATGC-3')

+ eukTF-RTPCR-3' (5'-CATGATCTTCATTCGCATCAG-3'). Primers were used in a final concentration of 400 nM. qRT-PCR runs were performed on a Rotorgene Q cycler (QIAGEN) using the 2x PerfeCTa SYBR Green SuperMix (Quanta BioSciences, Gaithersburg, MD, USA) and the following qRT-PCR conditions: initial denaturation step at 95˚C for 3 min sec, 45 cycles at 95˚C for 10 sec, 60˚C for 15 sec, and 72°C for 20 sec. Relative quantification was performed using the delta delta Ct method [38].

Z-Phe-Arg-AMC substrate assay

Purified recombinant SmCB2 (rSmCB2) expressed in *Pichia pastoris* was examined for its proteolytic activity towards its known fluorogenic substrate Z-FR-AMC as described before [35]. rSmCB2 was diluted and activated for 5 minutes at room temperature in citrate buffer (100 mM trisodium citrate, 2 mM DTT, pH 5.5). Z-Phe-Arg-AMC substrate (Bachem, Bubendorf, Switzerland) was dissolved at a concentration of 10 mM in dimethyl sulfoxide (DMSO) and diluted to 40 µM in citrate buffer (100 mM trisodium citrate, 2 mM DTT, pH 5.5). Z-Phe-Arg-AMC substrate (20 µM) in citrate buffer was added to the activated rSmCB2 to initiate the assay. Fluorescence was monitored for 30 minutes at 37°C on a Spectromax M2 fluorescence microplate reader (MTX Lab Systems, Bradenton, FL, USA) equipped with a 390 nm excitation filter and 460 nm emission filter. Initial rates of increase in fluorescence are represented as mean ± SD.

Statistical Analysis

Statistical analysis was performed with PRISM software (version 7.02; GraphPad) using a one-way ANOVA to test for differences between groups.

Results

Schistosoma mansoni preparations contain proteolytic activity towards FRETS-VWF73 substrate and multimeric VWF

FRETS-VWF73 substrate is a synthetic 73 amino acid peptide based on the amino acid sequence of the A2 domain of von Willebrand Factor (VWF). This fluorogenic substrate is used in a diagnostic assay (FRETS-VWF73 assay) to determine activity of ADAMTS13 [31], the peptidase that normally controls VWF multimer size through limited cleavage [11]. The FRETS-VWF73 peptide forms the minimal substrate for ADAMTS13, containing the ADAMTS13 cleavage site and the minimal sequence required for binding of ADAMTS13 [31]. We used the FRETS-VWF73 substrate as a starting point to determine whether *Schistosoma mansoni* contains peptidases that can cleave VWF, and could thereby mimic the controlling effects of ADAMTS13 on VWF. Different protein concentrations of *S. mansoni* worm homogenate (WH) (Figure

1A closed circles) or *S. mansoni* soluble worm fraction (SWF) (Figure 1B closed circles) were tested for their proteolytic activity on FRETS-VWF73 substrate. Both fractions of *S. mansoni* showed a dose-dependent proteolytic cleavage of FRETS-VWF73 substrate, indicating the presence of one or more VWF-cleaving peptidases in the fractions. The *S. mansoni* worms were isolated from the circulation of infected hamsters, therefore, in order to rule out contamination of the *S. mansoni* fractions with host ADAMTS13 from the hamster, both *S. mansoni* fractions were also tested in the presence of the peptidase inhibitor ethylenediaminetetraacetic acid (EDTA) (Figure 1A and B, open circles). EDTA inhibits a range of metallopeptidases, including ADAMTS13 [39](see also Figure 2B), through chelation of the metal ion required for proteolytic activity. Interestingly, analysis of the *S. mansoni* fractions in the presence of EDTA resulted in increased proteolytic activity towards FRETS-VWF73, thereby ruling out contaminating ADAMTS13 as the peptidase responsible for the observed cleavage of FRETS-VWF73.

Next, we examined whether *S. mansoni* fractions also have proteolytic activity towards native multimeric VWF. To allow proteolytic cleavage by ADAMTS13, VWF needs to be (partially) unraveled, either through sheer stress or through the addition of a denaturing agent, which is done in this experimental setup by the addition of urea [39]. Purified VWF was incubated with *S. mansoni* WH or pooled normal platelet-poor plasma (PNP) for 8 hours. Incubations were performed both in the presence and absence of EDTA, to exclude effects of possible contamination of the *S. mansoni* fractions with host ADAMTS13. Separation of the VWF multimers by gel electrophoresis under non-reducing conditions reveals the multimeric characteristic of VWF (Figure 1C). Cleavage of VWF results in the appearance of the slower and faster migrating satellite bands flanking the major VWF-multimer bands to form the so-called VWF triplets [40]. These satellite bands result from variation in the number of N-terminal fragments that the VWF-multimers contain after cleavage [40]. Uncleaved, purified VWF shows two bands after gel electrophoresis, corresponding to the slower migrating and intermediate triplet band. Incubation of VWF with PNP results in cleavage of VWF by ADAMTS13, which can be observed as the appearance of the faster migrating triplet band (black arrow).

Incubation with PNP in the presence of EDTA fully inhibits ADAMTS13 activity, and as a result only the slower migrating and intermediate triplet band can be observed. After incubation of VWF with *S. mansoni* WH, the faster migrating triplet band appears faintly. However, after incubation with WH in the presence of EDTA, the faster migrating triplet band appears strongly, indicating efficient cleavage of VWF by WH in the presence of EDTA. This efficient cleavage is also reflected by the loss of high molecular weight multimer bands of VWF. These results show that *S. mansoni* fractions contain a peptidase that cleaves multimeric VWF. Furthermore, cleavage of the FRETS-VWF73 substrate by *S. mansoni* fractions indicates that (at least one of) the cleavage site(s) of the peptidase may be located close to the ADAMTS13 cleavage site in the A2-domain of VWF.

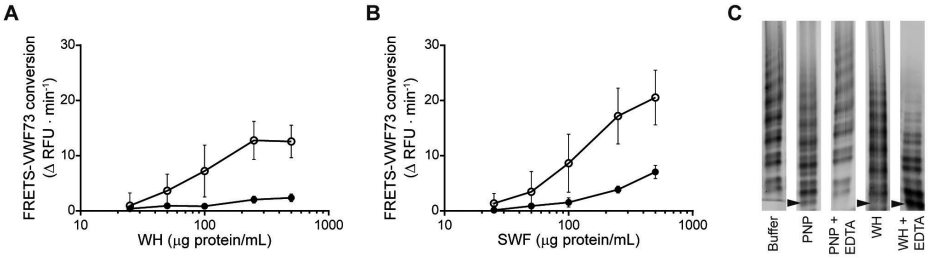

Figure 1. *S. mansoni* extracts contain a distinct VWF cleaving activity.

Cleavage of FRETS-VWF73 substrate was examined for different protein concentrations of *S. mansoni* worm homogenate (WH) (A) and *S. mansoni* soluble worm fraction (SWF) (B) in the presence (open circles) and absence (closed circles) of 5 mM EDTA. Initial rates of increase in fluorescence are represented as mean ± SD. All values represent triplicate independent experiments. C) Cleavage of native VWF by *S. mansoni* WH and pooled normal platelet-poor plasma (PNP) was examined with and without 5 mM EDTA. VWF multimers were incubated for 8 hours and separated by gel electrophoresis. Cleavage of VWF, characterized by appearance of the third triplet band, is indicated by black arrows (representative image of three independent experiments).

The VWF-cleaving peptidase belongs to the class of cysteine peptidases

Next, we characterized the VWF-cleaving peptidase of *S. mansoni* further to determine the class of peptidases it belongs to. Cleavage of the FRETS-VWF73 substrate was therefore examined in the presence of various peptidase inhibitors, each inhibiting a specific class of peptidases. N-ethylmaleimide (NEM) is an irreversible small-molecule inhibitor of cysteine peptidases. Leupeptin (Leu) is a reversible small-molecule inhibitor of a broad range of cysteine, serine, and threonine peptidase classes. Pepstatin A (PepA) is a reversible small-molecule inhibitor of aspartic peptidases. Phenylmethane sulfonylfluoride (PMSF) is an irreversible small-molecule inhibitor of serine peptidases and ethylenediaminetetraacetic acid (EDTA) is a chelating agent that inhibits metallopeptidases through chelation of the metal ion required for proteolytic activity. Proteolytic cleavage of FRETS-VWF73 by *S. mansoni* soluble worm fraction (SWF) was strongly inhibited by both NEM and leupeptin, but was unaffected by the addition of pepstatin A or PMSF (Figure 2A). This indicates that the VWF-cleaving peptidase most likely belongs to the class of cysteine peptidases. Additionally, it was verified whether addition of L-cysteine (L-cys) could enhance proteolytic cleavage. This is characteristic for members of the cysteine peptidase family as their proteolytic activity is dependent on reduction of the active site cysteine, which can be accomplished by addition of thiol-reducing agents, such as dithiothreitol or free cysteine. The indication that the VWF-cleaving peptidase belongs to the class of cysteine peptidases is indeed further strengthened by the observation that both EDTA and L-cysteine enhance proteolytic activity, which is characteristic behavior for (papain-like) cysteine peptidases [41,42].

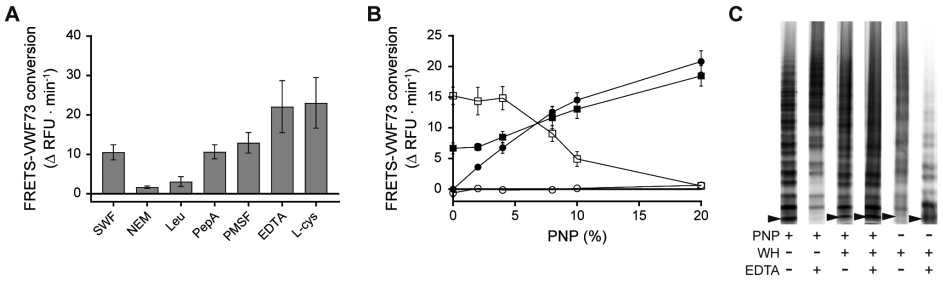

Figure 2. Characterization of the VWF-cleaving peptidase.

A) Cleavage of FRETS-VWF73 substrate by *S. mansoni* soluble worm fraction (SWF) (500 µg/mL) was studied in the presence or absence of various peptidase inhibitors (NEM 10 mM, leupeptin (Leu) 100 µM, pepstatin A (PepA) 5 µM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, EDTA 5 mM, L-cysteine (L-cys) 50 mM). Initial rates of increase in fluorescence are represented as mean ± SD. All values represent three replicates of duplo measurements. B) Cleavage of FRETS-VWF73 substrate by *S. mansoni* soluble worm fraction (SWF) (500 µg/mL) was studied in the presence or absence of pooled normal platelet-poor plasma (PNP) and/or EDTA in order to examine inhibition of *S. mansoni* cleavage of FRETS-VWF73 by plasma components. Closed circles (●) represent PNP, open circles (○) represent PNP with addition of EDTA, closed squares (■) represent *S. mansoni* soluble worm fraction (SWF) plus PNP, open squares (□) represent *S. mansoni* soluble worm fraction (SWF) plus PNP in the presence of EDTA. Initial rates of increase in fluorescence are represented as mean ± SD. All values represent three replicates of duplo measurements. C) Cleavage of native VWF by *S. mansoni* WH in the presence of PNP was examined with and without 5 mM EDTA. VWF multimers were incubated for 4 hours and separated by gel electrophoresis. Cleavage of VWF, characterized by appearance of the third triplet band, is indicated by black arrows.

Proteolytic activity of the VWF cleaving peptidase is partially inhibited by plasma components

As plasma contains many peptidase inhibitors, we next examined whether the schistosomal VWF-cleaving peptidase retained functionality in the presence of plasma components. Cleavage of FRETS-VWF73 substrate by *S. mansoni* soluble worm fraction (SWF) was examined in the presence of different amounts of PNP with or without EDTA (Figure 2B). PNP containing ADAMTS13 showed a dose-dependent cleavage of FRETS-VWF73 substrate (closed circles), which can be fully inhibited through the addition of EDTA (open circles). Measurement of a fixed concentration of *S. mansoni* soluble worm fraction (SWF) in the presence of increasing concentrations of PNP showed a large additional proteolytic effect of the *S. mansoni* peptidase at low plasma concentrations (closed squares). However, at plasma concentrations above 8% PNP there was no additional proteolytic effect of the *S. mansoni* peptidase on FRETS-VWF73. This does not reflect substrate depletion, as a further increase in the PNP concentration from 10 to 20% resulted in increased substrate conversion. Similarly, in the presence of EDTA the *S. mansoni* peptidase showed strong proteolytic cleavage of FRETS-VWF73 at low plasma concentrations, but not at higher plasma concentrations (open squares). These results indicate that human plasma contains components that can inhibit the VWF-cleaving peptidase of *S. mansoni* at higher plasma concentrations.

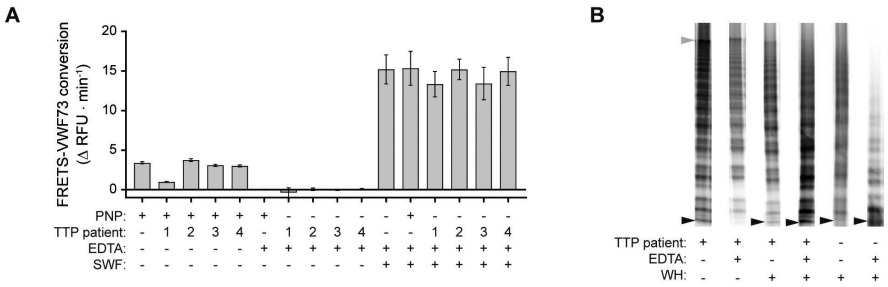

Figure 3. The *S. mansoni* VWF-cleaving peptidase retains partial activity in TTP patient plasma.

A) Cleavage of FRETS-VWF73 substrate by *S. mansoni* soluble worm fraction (SWF) (500 µg/mL) in the presence of 5 mM EDTA was studied in the presence or absence of 2% TTP patient platelet-poor plasma that has 0% ADAMTS13 activity, as determined before by FRETS-VWF73 substrate assay. Initial rates of increase in fluorescence are represented as mean ± SD. All values represent three replicates of measurements performed in duplicate. Statistical analysis was performed with one-way ANOVA and Differences were considered statistically significant when $p \leq 0.05$. B) Cleavage of native VWF by *S. mansoni* WH in the presence of TTP patient platelet-poor plasma was examined with and without 5 mM EDTA. VWF multimers were incubated for 2 hours and separated by gel electrophoresis. Cleavage of VWF, characterized by appearance of the third triplet band, is indicated by black arrows. Very high multimers that are characteristic for TTP patient plasma are indicated by the grey arrow.

We next verified whether the *S. mansoni* peptidase is also able to cleave native VWF in the presence of human plasma components. Purified VWF was incubated with both *S. mansoni* WH and PNP (3.6%) in the presence or absence of EDTA, followed by analysis of VWF multimers through gel electrophoresis (Figure 2C). The occurrence of cleavage of native VWF by the *S. mansoni* peptidase at low plasma concentrations (3.6%), with and without addition of EDTA, was consistent with the observed cleavage of FRETS-VWF73 at low plasma concentrations. Also in this experiment plasma components seemed to partially inhibit the *S. mansoni* peptidase, as cleavage of multimeric VWF in the presence of EDTA occurs more efficiently in the absence of PNP, which is visible by the increased disappearance of high multimer bands in the absence of PNP.

The VWF cleaving peptidase retains activity in TTP patient plasma

Insufficient cleavage of VWF in humans leads to the development of thrombotic thrombocytopenic purpura (TTP), a rare but severe form of thrombotic microangiopathy caused by clumping of platelets by ULVWF [15,16]. A deficiency of ADAMTS13 is associated with the development of TTP. This ADAMTS13 deficiency can be congenital, caused by mutations in the ADAMTS13 gene, or more often idiopathic, caused by inhibition of ADAMTS13 activity or enhanced clearance through binding autoantibodies directed at ADAMTS13 [43,44]. After we discovered that *S. mansoni* soluble worm fraction contains a VWF-cleaving peptidase, we next examined whether this peptidase would be functional in TTP patient plasma, or whether it would be affected by specific components of the

TTP patient plasma such as autoantibodies. Cleavage of FRETS-VWF73 substrate by *S. mansoni* soluble worm fraction in the presence of EDTA was therefore examined with addition of 2% PNP or platelet-poor plasma obtained from 4 different TTP patients that had 0% ADAMTS13 activity as previously determined by FRETS-VWF73 assay (patient data not shown) (Figure 3A). ADAMTS13 activity in PNP was strongly inhibited after addition of plasma from TTP patient 1. This would indicate the presence of autoantibodies against ADAMTS13 in plasma from this TTP patient, which interferes with the ADAMTS13 activity from PNP. In contrast, no significant differences in VWF cleavage of FRETS-VWF73 by *S. mansoni* soluble worm fraction were observed upon addition of TTP patient plasma, indicating that the VWF-cleaving *S. mansoni* peptidase is not sensitive to components specific for TTP patient plasma at low plasma concentrations.

We next examined whether the *S. mansoni* peptidase could cleave multimeric VWF in TTP patient plasma. Purified VWF was incubated with both *S. mansoni* WH and TTP patient plasma (3.6%) in the presence or absence of EDTA, followed by analysis of VWF multimers through gel electrophoresis (Figure 3B). The occurrence of cleavage of native VWF by the *S. mansoni* peptidase in the presence of TTP patient plasma (3.6%) with and without addition of EDTA, is consistent with the observed cleavage of FRETS-VWF73 in the presence of TTP patient plasma. Also in this experiment plasma components seemed to partially inhibit the *S. mansoni* peptidase, as cleavage of multimeric VWF in the presence of EDTA occurs more efficiently in the absence of TTP patient plasma, which is visible by the increased disappearance of high multimer bands in the absence of TTP patient plasma.

Anion exchange chromatography fractionation combined with mass spectrometry identifies SmCB2 as a candidate gene for the *S. mansoni* VWF-cleaving cysteine peptidase

Anion exchange chromatography fractionation was used to separate proteins of *S. mansoni* in order to obtain fractions with high proteolytic activity towards the fluorogenic FRETS-VWF73 substrate. The three fractions with the highest activity in the FRETS-VWF73 substrate assay were pooled and subsequently analyzed by mass spectrometry. Proteins present in the active fraction were compared to the protein content of the flow-through fraction (control fraction) containing low VWF-cleaving activity at equal protein concentrations. Five cysteine peptidases were identified in the active fraction (Table 1), however, only one of these cysteine peptidases was consistently enriched in both mascot ion score and peptide spectrum matches (PSM) in the active fraction compared to the control fraction throughout three independent experiments. This cysteine peptidase, *Schistosoma mansoni* cathepsin B (Smp_141610), showed 17.2 ± 2.3 fold enrichment in score and 12.4 ± 3.1 fold enrichment in PSM compared to the control fraction. This identified the *S. mansoni* cathepsin B gene as

the prime candidate to encode the *S. mansoni* VWF-cleaving peptidase.
S. mansoni cathepsin B (Smp_141610) was first isolated and characterized by Caffrey *et al.* [35]. This cathepsin B peptidase was named *S. mansoni* cathepsin B2 (SmCB2, Uniprot Q95PM1) to distinguish it from the first recorded *S. mansoni* cathepsin B, SmCB1 [35,45](Uniprot AAA29865). Consistent with this nomenclature, we will here refer to the candidate gene *S. mansoni* cathepsin B (Smp_141610) as SmCB2.

Table 1: Cysteine peptidases present in the schistosomal fraction with proteolytic activity towards FRETS-VWF73 substrate obtained after anion exchange chromatography of *S. mansoni* soluble worm fraction

Accession	Product	MW (kDa)	Ratio Score A	Ratio Score B	Ratio Score C	**Average Ratio score**	SD Ratio Score	Ratio PSM A	Ratio PSM B	Ratio PSM C	**Average PSM score**	SD Ratio PSM
Smp_141610.1	cathepsin b	39.1	15.7	16.1	19.9	**17.2**	2.3	12.2	9.4	15.5	**12.4**	3.1
Smp_179170.1	hemoglobinase (C13 family)	22.2	0.9	0.8	1.6	**1.1**	0.4	0.9	0.8	1.4	**1.0**	0.3
Smp_075800.1	hemoglobinase	49.1	0.7	0.8	1.4	**1.0**	0.4	0.7	0.7	1.4	**0.9**	0.4
Smp_067060.1	Cathepsin B1 isotype 2	39.2	0.5	0.3	0.9	**0.6**	0.3	0.4	0.3	1.0	**0.6**	0.4
Smp_103610.1	cathepsin B-like cysteine proteinase	38.5	0.3	0.2	0.8	**0.4**	0.3	0.3	0.2	0.9	**0.5**	0.4

Score: mascot ion score; PSM: peptide spectrum matches (total number of identified peptide spectra matched for the protein); A, B, and C indicate three independent experiments.

SmCB2 cleaves VWF

The expression of SmCB2 was decreased in adult male *S. mansoni* worms using RNA interference in order to investigate the effect of decreased SmCB2 expression on cleavage of VWF by *S. mansoni* soluble worm fraction. Male *S. mansoni* worms were incubated for four days with siRNA molecules directed against the candidate SmCB2 gene. Additionally, control worms were prepared that were not incubated with siRNA. Gene expression after RNAi was determined by qRT-PCR for the candidate gene SmCB2 and two control genes (Figure 4A). Control genes that were selected were *S. mansoni* cathepsin S (Smp_139240.1) and another cathepsin B gene (Smp_085180). The successful siRNA treatment with siRNA A, siRNA B, or a combination of both, reduced the relative gene expression of the candidate gene below 12% compared to the control, without affecting gene expression of the two control genes. Subsequently, proteolytic activity of the soluble worm fraction of the siRNA treated worms towards FRETS-VWF73 substrate was examined (Figure 4B). No significant differences in proteolytic activity towards FRETS-VWF73 substrate were found between the control treated worms and the RNAi treated worms. This demonstrates that the reduced mRNA expression of the selected

candidate SmCB2 did not result in a reduced VWF-cleaving peptidase activity, which can be explained by either a very long half-life of the SmCB2 protein or by the presence of redundancy by other *schistosomal* VWF-cleaving peptidases.

To directly investigate the capacity of SmCB2 to cleave VWF, recombinant expressed SmCB2 (rSmCB2) in *Pichia pastoris* was purified and examined for its proteolytic activity towards its known fluorogenic substrate Z-FR-AMC as described before [35] and towards the FRETS-VWF73 substrate. As shown in Figure 5, purified rSmCB2 is an active peptidase that can cleave the FRETS-VWF73 substrate in a dose dependent manner, which demonstrates that SmCB2 is a schistosomal peptidase that can cleave the same VWF domain as ADAMTS13. Interestingly, the proteolytic activity of rSmCB2 towards the FRETS-VWF73 substrate was not enhanced by the presence of EDTA, which could suggest that SmCB2 is not the only schistosomal peptidase that can cleave human VWF or that addition of EDTA to soluble worm fraction results in activation of SmCB2 in crude homogenates by for instance induction of cleavage of inhibiting pro-peptides or conformational changes that remove other inhibitory factors.

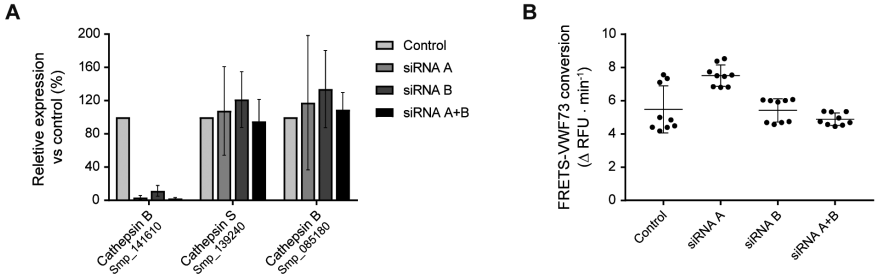

Figure 4. Proteolytic activity towards FRET-VWF73 substrate in *S. mansoni* worms was not affected by RNA-interference of cathepsin B2.

RNA interference (RNAi) was performed using two different siRNA molecules (A and B) directed against *S. mansoni* cathepsin B2 (SmCB2, Smp_141610) or a mixture of both (A+B). Negative control worms were electroporated without siRNA addition. A) Relative expression of SmCB2 and two control genes (Cathepsin S (Smp_139240) and a different Cathepsin B gene (Smp_085180)) was determined by RT-PCR and normalized to eukaryotic translation factor (Smp_008900) as a reference gene. Relative expression versus the negative control was determined in triplicate and is represented as mean ± SD. B) Cleavage of FRETS-VWF73 substrate by siRNA treated *S. mansoni* was determined in the presence of 5 mM EDTA. Soluble worm fraction was prepared from the different siRNA treated worms (siRNA A, siRNA B, or siRNA A+B) and from control worms that underwent electroporation but did not receive siRNA. Initial rates of fluorescence are represented as mean ± SD. SiRNA incubations were performed in triplicate and FRETS-VWF73 cleavage was determined in triplicate for each RNAi sample.

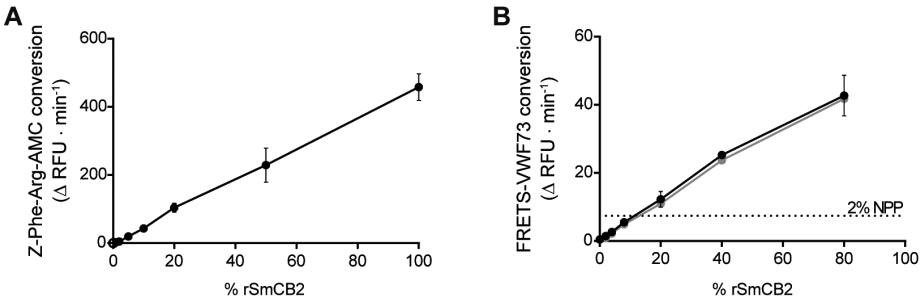

Figure 5. Proteolytic activity towards FRET-VWF73 substrate of purified recombinant SmCB2.

Purified recombinant SmCB2 (rSmCB2) was examined for proteolytic activity towards two fluorescent substrates Z-Phe-Arg-N AMC (A) and FRETS-VWF73 with and without 5 mM EDTA (B, black and gray line respectively). The dashed line in panel B represents the VWF-cleavage activity of normal pooled plasma at a 2% (v/v) concentration. Initial rates of fluorescence are represented as mean ± SD. Substrate cleavage was determined in triplicate for a serial dilution of rSmCB2.

Discussion

Here, we report the finding of a peptidase of *Schistosoma mansoni* that cleaves human von Willebrand Factor (VWF). This peptidase displays proteolytic activity towards FRETS-VWF73 substrate, containing part of the A2 domain of VWF, and towards purified multimeric VWF. The peptidase belongs to the class of divalent cation-independent cysteine peptidases and thereby differentiates itself from currently known human peptidases that cleave VWF, which either belong to the class of the metalloproteinases, such as ADAMTS13, MMP-8 and MMP-9, or the class of serine peptidases, such as plasmin, the leukocyte-derived proteinase 3, neutrophil elastase and cathepsin G, or require divalent cations for their catalytic activity, such as calpain.

When examining the peptidase activity present in the soluble worm fraction of *S. mansoni* in a plasma environment, we observed partial inhibition of the peptidase in the presence of plasma components. The inhibition of peptidase activity by plasma components is not surprising, as plasma contains many peptidase inhibitors. Moreover, peptidase inhibitors constitute the third largest group of functional plasma proteins by weight, after albumin and immunoglobulins [46]. However, this inhibition by plasma components was observed under the non-physiological acidic assay conditions required for the *in vitro* assessment of VWF-cleavage activity, as is the case for ADAMTS13 [47]. Under physiological conditions, in which the A2 domain of VWF is unraveled by shear stress instead of non-natural conditions, the observed inhibition by plasma factors could be different.

Using anion exchange chromatography fractionation in combination with mass spectrometry to characterize the protein composition in the fractions comprising

VWF-cleavage activity, SmCB2 was identified as the prime candidate peptidase of *S. mansoni* that could cleave human VWF. Subsequently, purified recombinant SmCB2 was shown to be able to cleave the A2 domain of VWF in the same small peptide region as ADAMTS13, which confirmed that SmCB2 is a schistosomal cysteine peptidase that can cleave human VWF.

SmCB2 is a well-studied cysteine peptidase of *S. mansoni* [35]. Localization studies on SmCB2 have demonstrated that the peptidase is mainly localized in the dorsal and lateral tubercles of the tegument of male worms. Because of this localization in the outer-surface structure of the worm, it was suggested that SmCB2 should have a function at the host-parasite interface, such as turnover of tegumental or endocytosed proteins or a protective capacity [35].

SmCB2 has been shown to cleave both the Z-Phe-Arg-NHMec, a Cathepsin L/B substrate, and Z-Arg-Arg-NHMec, a cathepsin-B selective substrate, which both have an Arg at the S1 position [35]. Interestingly, the sequence of the FRETS-VWF73 substrate, used to assess proteolytic activity towards VWF contains no arginine residues between its fluorophore and quencher [31]. Substrate specificity for papain-like endopeptidases, such as SmCB2 is, however, primarily defined by the residue in the S2 position[35]. SmCB2 has been demonstrated to have a preference for Phe > Leu > Val>> Arg at the S2 position, and it has been suggested that SmCB2 has a less restricted S2 specificity compared to the mammalian cathepsin B enzyme [35]. While the FRETS-VWF73 substrate does not contain Phe or Arg residues between its fluorophore and quencher, both Leu and Val residues are present [31].

Assessment of the pH profile of SmCB2 has shown that the pH optimum of SmCB2 is approximately at pH value 5.0 – 5.5, whereas the enzyme is reported to be inactive at a pH value above 6.5 [35]. This acidic pH optimum is similar to that reported for ADAMTS13, which is also incapable to cleave VWF at physiological pH *in vitro* [47]. Cleavage of native VWF by schistosomal peptidases was assessed at a pH-value between pH 7.0 and pH 8.0, suggesting that schistosomal peptidases would be able to cleave native VWF at physiological pH. Further assessment of VWF cleavage by SmCB2 in assays with more physiological assay conditions or in an *in vivo* setting is therefore vital in the characterization of SmCB2 as a *S. mansoni* VWF-cleaving peptidase. In addition to a potential function for SmCB2 in survival of the parasite within its host, we also examined whether the discovery of this VWF-cleaving peptidase could be beneficial in a different setting. Insufficient cleavage of VWF results in a rare but severe form of thrombotic microangiopathy, called thrombotic thrombocytopenic purpura (TTP) [15,16]. TTP is associated with a deficiency in ADAMTS13 activity which leads to accumulation of ULVWF and subsequent platelet aggregation. The ADAMTS13 deficiency in TTP can be congenital, but is in most cases idiopathic, caused by inhibition of ADAMTS13 activity or enhanced clearance through

autoantibodies directed at ADAMTS13 [43,44]. Currently, the first-line treatment of TTP is daily therapeutic plasma exchange, in order to restore the ADAMTS13 activity and potentially also to remove ADAMTS13 immune complexes, high-molecular-weight VWF multimers and inflammatory cytokines [44]. Additionally to therapeutic plasma exchange, corticosteroids, rituximab, or other immunomodulators can be used to treat TTP patients [44,48]. Also, clinical trials are ongoing to test the use of recombinant ADAMTS13 in the treatment of TTP [48–50]. The use of VWF-cleaving peptidases, such as the here discovered SmCB2 could potentially form interesting additions to the current treatment strategies for TTP patients. Especially because we showed that the *S. mansoni* VWF-cleaving peptidase is not affected by inhibitory autoantibodies directed at ADAMTS13, which would be advantageous over recombinant ADAMTS13. This makes SmCB2 an interesting candidate for alternative control of VWF functionality in TTP patients. The therapeutic effect for TTP patients of SmCB2, as well as its capacity to cleave VWF under physiological conditions, will be examined In future studies using existing animal models for TTP [51].

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5

CHAPTER 5

Truncation of ADAMTS13 by plasmin enhances its activity in plasma

Chantal C. Clark[1,‡], Mirjam M. Mebius[1,2,‡], Steven de Maat[1], Aloysius G. M. Tielens[2,3], Philip G. de Groot[1], Rolf T. Urbanus[1], Rob Fijnheer[1], Bouke P. C. Hazenberg[4], Jaap J. van Hellemond[2], Coen Maas[1]

‡ Authors contributed equally

[1] Department for Clinical Chemistry & Haematology, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands

[2] Department of Medical Microbiology & Infectious Diseases, Erasmus University Medical Center, Rotterdam, the Netherlands

[3] Department for Biochemistry & Cell Biology, Faculty Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

[4] Department of Rheumatology & Clinical Immunology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

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Abstract

ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) cleaves von Willebrand Factor (VWF) multimers to control their thrombogenicity. The fibrinolytic enzyme plasmin can cleave VWF in a similar manner. However, plasmin can also cleave ADAMTS13, which ultimately inactivates it. This leaves the overall role of plasmin in primary haemostasis uncertain.

We investigated the combined molecular effects of plasmin on VWF and ADAMTS13. We first identified that plasmin destroys FRETS-VWF73 substrate by cleaving the ADAMTS13 binding region in a buffered system. We next investigated how plasmin affects both VWF and ADAMTS13 under static conditions in plasma by western blotting. We found that globular VWF is largely protected from plasmin cleavage. However, ADAMTS13 is rapidly cleaved under these conditions, suggesting inactivation. Surprisingly, we observed that plasmin enhances ADAMTS13 activity in a modified two-stage FRETS-VWF73 assay that protects FRETS-VWF73 substrate from degradation. In direct binding studies under the same conditions, we found that plasmin generates multiple C-terminally truncated forms of ADAMTS13 with VWF-binding capacity. In an effort to seek evidence for this mechanism *in vivo*, we analysed plasma from patients with systemic amyloidosis, which is hallmarked by a hyperfibrinolytic state. We found that their plasma contained increased levels of C-terminally truncated forms of ADAMTS13, which correlated with their hyperfibrinolytic state.

We propose that truncation of ADAMTS13 by plasmin abolishes intramolecular self-association, which improves interaction with unfolded VWF.

Introduction

The metalloproteinase ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) cleaves von Willebrand Factor (VWF) multimers to control their thrombogenicity. As a result, low or absent ADAMTS13 activity is associated with thrombotic thrombocytopenic purpura (TTP), a rare form of thrombotic microangiopathy [1]microangiopathic haemolytic anaemia and a variable degree of ischaemic organ damage, particularly affecting the brain, heart and kidneys. Acute TTP was almost universally fatal until the introduction of plasma therapy, which improved survival from <10% to 80-90%. However, patients who survive an acute episode are at high risk of relapse and of long-term morbidity. A timely diagnosis is vital but challenging, as TTP shares symptoms and clinical presentation with numerous conditions, including, for example, haemolytic uraemic syndrome and other thrombotic microangiopathies. The underlying pathophysiology is a severe deficiency of the activity of a disintegrin and metalloproteinase with thrombospondin motifs 13 (ADAMTS13. TTP attacks are accompanied by plasminogen activation [2]. We previously found preclinical evidence for a protective role of plasmin in TTP: it can degrade platelet-VWF complexes and stimulation of plasmin activity has therapeutic value in a mouse model for TTP [2]. However, unrestricted exposure of ADAMTS13 to plasmin in a buffered system destroys its activity [3]. These seemingly opposite functions of plasmin leave its overall contribution in physiological primary haemostasis unclear. In the present study, we aimed to obtain insight into the effects that plasmin simultaneously exerts on both VWF and ADAMTS13 under static conditions in plasma. A monoclonal antibody mapping study previously showed that plasmin cleaves VWF into three fragments. This indicates the presence of two distinct cleavage sites, rather than random cleavage after one of the 108 lysine residues (4% of all residues) in a VWF monomer [4]. One of the plasmin cleavage sites was mapped to a region within VWF A2 domain (1476-1643 numbering includes pro-peptide), where the ADAMTS13 cleavage site is also located (1605-1606 numbering includes pro-peptide) [4]. This helps to explain our finding that plasmin cleaves VWF in a conformation-dependent manner similar to ADAMTS13 [2]. The diagnostic ADAMTS13 activity assay (FRETS-VWF73 assay) uses a synthetic peptide based on the VWF A2 domain, including the ADAMTS13 cleavage site, flanked by a fluorophore and quencher. (Figure 1A) [5] ADAMTS13, cleaves von Willebrand factor (VWF. The long sequence that follows the quencher is required for ADAMTS13 binding, and subsequent cleavage [5]ADAMTS13, cleaves von Willebrand factor (VWF. In our study, we first investigated the effect of plasmin on FRETS-VWF73 substrate and VWF in plasma. Subsequently we investigated the effect of plasmin on ADAMTS13 activity and structure.

Material and Methods

Reagents

The following reagents were used: normal pooled platelet-poor citrated plasma (hereafter called plasma; 50 healthy donors, Sanquin, the Netherlands); FRETS-VWF73 substrate (AnaSpec, Fremont, California, United States); plasmin substrate (I-1390; Bachem, Bubendorf, Switzerland); rabbit polyclonal anti-VWF (A0082; DAKO, Carpinteria, California, United States); goat polyclonal anti-ADAMTS13 (A300-391A; Bethyl, Montgomery, Texas, United States); goat polyclonal anti-plasminogen (GAPG-AP; Affinity Biologicals, Ancaster, ON, Canada); donkey-anti-goat IR dye 800 (926-32214; LI-COR, Lincoln, Nebraska, United States); goat-anti-rabbit Alexa Fluor 680 (A21076; Thermo Fisher Scientific, Waltham, Mississippi, United States); streptokinase (CSL Behring, Marburg, Germany); aprotinin (Sigma-Aldrich, St Louis, Missouri, United States); PPACK (D-Phenylalanine-Proline-Arginine Chloromethyl Ketone; Haemtech, Essex Junction, Vermont, United States); hirudin (Pharmion, Tiel, the Netherlands); bovine serum albumin (BSA; Sigma-Aldrich) and purified VWF (Biotest AG, Dreieich, Germany). Plasminogen was purified from plasma as previously described [6].

In silico prediction of cleavage sites

Cleavage sites for trypsin-like serine proteases in FRETS-VWF73 substrate were predicted with ExPASy PeptideCutter software [7].

Direct effects of plasmin on FRETS-VWF73 substrate

We investigated the influence of plasmin on FRETS-VWF73 substrate in a two-stage experimental setup. FRETS-VWF73 substrate was dissolved at a concentration of 100 µM in 25% (v/v) dimethyl sulfoxide (DMSO) and diluted to 4 µM in assay buffer (5 mM Bis-Tris, 25 mM $CaCl_2$, 0.005% Tween-20, pH 6.0) prior to experiments. Plasminogen (1.2 mg/mL) was activated with streptokinase (50 U/mL) for 30 minutes at 37°C in HEPES-buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, pH 7.4).

Stage 1: To investigate whether plasmin cleaves between the fluorophore and quencher of FRETS-VWF73 substrate, we incubated 0.2 nmol FRETS-VWF73 in 30 µL assay buffer with 20 µL streptokinase-activated plasminogen in HBS. To prevent coagulation of plasma at a later stage in the experiment, 100 U/mL hirudin (final concentration) was added. The resulting pH of these 50 µL reactions was 6.85. Buffer controls without streptokinase-activated plasminogen were included in the experiments. Generation of fluorescence was monitored for 10 minutes at 30°C in a 96-well white MaxiSorp microtiter plate (Thermo Scientific, Roskilde, Denmark) on a SpectraMax M^2 fluorescence microplate reader (MTX Laboratory Systems, Bradenton, Florida, United States) equipped with a 340 nm excitation filter and 450 nm emission filter. The reaction rate was calculated by linear regression analysis using PRISM software (version 7.02; GraphPad, San Diego, California, United States).

Stage 2: We next assessed whether FRETS-VWF73 substrate remains sensitive to plasma ADAMTS13 after exposure to plasmin. After following the procedure of *stage 1*, we subsequently added 10 µL of inhibitor cocktail in HBS to all samples (100 µM PPACK, 250 KIU/mL aprotinin; final in-assay concentrations) and incubated for 10 minutes at 30°C to block plasmin activity. Subsequently, 30 µL of assay buffer was added. Finally, 10 µL of diluted plasma in HBS (20% v/v) was added to initiate the assay. In this way, the ratio between assay buffer and HBS (and resulting pH of 6.85) was maintained between the two stages of the experiment. Generation of fluorescence was monitored for 1 hour at 30°C on the SpectraMax M^2 fluorescence microplate reader.

Effects of plasmin on ADAMTS13 activity

The direct influence of plasmin on ADAMTS13 activity in plasma was studied by activating plasminogen in 100 µL plasma by adding 25 µL streptokinase in HBS (1,000 U/mL; final concentration) or HBS (buffer control) for 30 minutes at 37 °C. Next, ADAMTS13 activity was measured by FRETS-VWF73 assay as published with minor modifications [5]ADAMTS13, cleaves von Willebrand factor (VWF. In brief, plasma was diluted in assay buffer (2% v/v, final dilution) and FRETS-VWF73 substrate (0.2 nmol) was added to initiate the assay and fluorescence was measured for 1 hour at 30°C. The reaction volume of this experiment consists for 97.5% of assay buffer (pH 6.0), 2% plasma and 0.5% HBS (accommodates streptokinase). In further experiments, plasmin activity was inhibited prior to determination of plasma ADAMTS13 activity by addition of 10% (v/v) inhibitor cocktail in HBS and 100 U/mL hirudin (final concentration) and incubation for 10 minutes at 37°C. Next, 2% (v/v) plasma in assay buffer supplemented with inhibitor cocktail was incubated with FRETS-VWF73 substrate (0.2 nmol) to initiate the assay. Fluorescence was measured for 1 hour at 30°C. The reaction volume of this experiment consists for 90% of assay buffer (pH 6.0), 8% HBS (accommodates streptokinase and inhibitor cocktail) and 2% plasma.

Plasmin activity assay

In control experiments, we confirmed activation and inhibition of plasma purified plasminogen under the conditions as described in 'direct effects of plasmin on FRETS-VWF73 substrate' and of plasma plasminogen under the conditions as described in 'Effects of plasmin on ADAMTS13 activity'. Hereto, samples were diluted 20-fold in HBS in 96-well white MaxiSorp plates. Where indicated, HBS was supplemented with inhibitor cocktail to demonstrate efficacy of plasmin inhibition. A final concentration of 12.5 µM I-1390 (plasmin substrate) was added and fluorescence was measured at 37 °C with excitation at 380 nm and emission at 460 nm. Initial reaction rates were calculated by linear regression analysis.

SDS-PAGE and immunoblotting

Plasminogen, ADAMTS13, and VWF were investigated by immunoblotting 0.5 µL of plasma in which plasminogen had been activated and inhibited as described in 'Effects of plasmin on ADAMTS13 activity'. Plasma samples were diluted 43-fold in sample buffer (62.3 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.001% bromophenol blue) and supplemented where indicated with 8.3 mM dithiothreitol (DTT) for reduction. Samples were boiled at 95°C for 10 minutes and 30 µL (containing 0.5 µL of original plasma) was separated by SDS-PAGE on 7.5% gels. For separation of ADAMTS13 and plasminogen under reducing conditions, 4 to 12% gradient gels were used. Proteins were transferred to immobilon-FL polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Tullagreen, Ireland) and blocked with 1:1 odyssey blocking buffer (LI-COR) and Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 7.4), followed by overnight incubation with the anti-ADAMTS13, anti-plasminogen or anti-VWF, diluted 1:2,500 at 4 °C. The anti-ADAMTS13 antibody is affinity purified using an epitope specific to ADAMTS13 mapping between residues 250 and 350 (part of the metalloproteinase and disintegrin domain) using the numbering given in entry NP_620594.1 (GeneID 11093). Membranes were subsequently incubated with donkey-anti-goat IR dye 800 (for anti-ADAMTS13 and anti-plasminogen) or goat-anti-rabbit Alexa Fluor 680 antibody (for anti-VWF) diluted 1:10,000 for 2 hours at room temperature. Membranes were scanned on a near-infrared scanner (Odyssey) and band intensities were quantified with Odyssey V3.0 software (LI-COR).

ADAMTS13 binding to immobilized VWF

Binding of ADAMTS13 to immobilized VWF in control and streptokinase activated and inhibited plasma (prepared as described in the section 'Effects of plasmin on ADAMTS13 activity') was investigated as published with minor modifications [8]a metalloprotease, cleaves von Willebrand factor (VWF. Ninety-six-wells Nunc-Immuno MaxiSorp microtiter plates (Thermo Scientific) were coated with VWF overnight at RT (100 µL/well; 10 µg/mL VWF in phosphate buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 9.2 mM $Na_2HPO_4 \cdot H_2O$, 1.76 mM KH_2HPO_4, pH 7.4]) and blocked with 200 µL/well blocking buffer (PBS, 2.5% BSA, 0.1% Tween-20) for 1 hour at RT. Where indicated, plasmin activity was triggered and terminated in plasma samples prior to binding experiments (as described in the section 'Effects of plasmin on ADAMTS13 activity'), buffer controls were included to correct for sample dilution. All plasma samples were supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA; to block ADAMTS13 activity). A sample of 100 µL was incubated in each well for 2 hours at RT. Next, the wells were washed three times with PBS 0.1% Tween-20 and bound ADAMTS13 was eluted in three times the sample buffer. The eluted ADAMTS13 from 48 wells per condition was combined and equally divided into a non-reduced, and a reduced sample (to which DTT was added) prior to analyses.

Patient plasma samples

Citrated plasma was collected from systemic amyloidosis patients and controls without systemic amyloidosis (i.e., localized amyloidosis) at Groningen University Medical Center under institutional ethical approval and informed consent as described earlier [9,10]AL type in 80, hereditary ATTR type in 27, and localized amyloidosis in 22 cases. SAP scintigrams were obtained 24 hours after tracer injection and were analyzed for abnormal patterns of uptake. Sensitivity and specificity were determined, and scintigraphic findings were compared with clinical data. RESULTS Diagnostic sensitivity of SAP scintigraphy for systemic AA, AL, and ATTR amyloidosis was 90%, 90%, and 48% respectively, and specificity was 93%. The distribution of amyloid was less diverse in AA than in AL type. Myocardial uptake was not visualized in any patient. Splenic amyloid was very frequent (80%. Selection was based on positive Congo red biopsies and number and location of sites with amyloid depositions. In a previous study, plasmin-α2 antiplasmin (PAP) complex levels were determined in duplicate within 5 months using a commercially available ELISA (Technoclone GmbH, Vienna, Austria, Cat. No. TC11060), according to the manufacturer's instructions [11]. ADAMTS13 activity was determined as described earlier. The binding of ADAMTS13 in patient plasma to immobilized VWF was investigated by incubating 30 µL plasma samples (undiluted) for more than 2 hours on immobilized VWF. Per patient six wells were combined and analyzed on western blot. Band intensities of uncleaved 190-kDa and truncated 170-kDa ADAMTS13 products were quantified by densitometry with Odyssey V3.0 software (LI-COR). The fraction of truncated ADAMTS13 was expressed relative to the fraction of uncleaved ADAMTS13 in individual patients.

Statistical analysis

Statistical analysis was performed with PRISM software (version 7.02; GraphPad) using Wilcoxon matched-pairs signed rank test or Friedman test with Dunn's multiple comparisons test. Correlation was tested using non-parametric Spearman's correlation. Results were statistically significant when $p \leq 0.05$.

Results

The ADAMTS13 binding tail of FRETS-VWF73 substrate is sensitive to plasmin cleavage

ExPASy PeptideCutter software predicts that the FRETS-VWF73 sequence contains five cleavage sites for trypsin-like enzymes [7]. Four of these are located in the sequence that mediates ADAMTS13 binding (Figure 1A), but no cleavage sites are predicted between the fluorophore and quencher.

We exposed FRETS-VWF73 substrate to different concentrations of purified streptokinase-activated plasminogen (plasmin activity was confirmed in Supplementary Figure S1). We found that no fluorescence was generated (Figure 1B), confirming the sequence between fluorophore and quencher does not contain a plasmin cleavage site [12,13]. When FRETS-VWF73 was exposed to plasmin activity for 10 minutes and subsequently blocked with inhibitor cocktail (efficacy confirmed in Supplementary Figure S1B), its functionality to act as a substrate for plasma ADAMTS13 was dose dependently impaired. (Figure 1C; grey circles). The inhibitor cocktail protects the substrate from plasmin-mediated destruction, which eliminates potential analytical threats in our next experiments (Figure 1C; black circles).

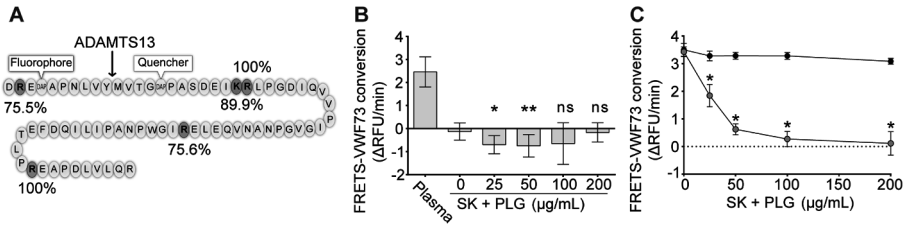


Figure 1. Plasmin cleaves the ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) binding tail of FRETS-VWF73 substrate in buffer.

(A) Predicted cleavage sites with a probability of greater than 75% for trypsin-like serine proteases in FRETS-VWF73 substrate (highlighted K and R residues) and the cleavage site for ADAMTS13 (indicated by arrow). (B) FRETS-VWF73 substrate conversion in the presence of a concentration series of streptokinase (SK)-activated plasminogen (PLG). As a positive control, FRETS-VWF73 substrate conversion by plasma ADAMTS13 is shown (indicated by plasma). (C) FRETS-VWF73 substrate functionality towards plasma ADAMTS13 was tested after pre-exposure to plasmin, which was terminated with inhibitor cocktail prior to analysis (grey circles). In control experiments, we found that inhibitor cocktail protects the substrate from plasmin-mediated destruction (black circles). (B,C) Initial rates of fluorescence as mean ± standard deviation (SD) of three separate experiments, performed in duplicate. (B) Statistical analysis was performed with Friedman's test with Dunn's multiple comparisons test where all values are compared with 0 μg/mL plasmin. (C) Statistical analysis was performed with Wilcoxon matched-pairs signed-rank test. Results were considered statistically significant when $p \leq 0.05$ (ns, non-significant; *$p \leq 0.05$; ** $p \leq 0.01$).

Plasmin predominantly cleaves ADAMTS13 in plasma under static conditions

We next triggered plasmin activity in plasma with streptokinase (confirmed in Supplementary Figure S2), terminated its activity with inhibitor cocktail and investigated VWF and ADAMTS13 by immunoblotting under reduced conditions. We found that VWF monomer content did not significantly diminish after plasmin activity (Figure 2A and B), although a 176-kDa cleavage product appeared [4]. This indicates that globular VWF

is largely resistant against plasmin cleavage, which fits well with the earlier reported shear-dependent cleavage by us [2] and others [12]. Under the same conditions, the ADAMTS13 band at 190 kDa was consumed (-78.7 ± 13.2%, P = 0.0039; Figure 2C and D) and there was some evidence for the generation of truncated products at approximately 130 and 90 kDa (Figure 2C). These findings together show that under static conditions in plasma, plasmin favors cleavage of ADAMTS13 over globular VWF.

Plasmin cleavage enhances ADAMTS13 activity in plasma

It was previously reported that plasmin cleaves recombinant ADAMTS13 [3]. In a buffered system, this cleavage is extensive and ultimately results in ADAMTS13 inactivation [3]. It is noteworthy that the first stages of plasmin cleavage take place at the C-terminus, resulting in truncated products with an intact metalloprotease domain. In hyperfibrinolytic (α2-antiplasmin deficient) plasma, cleavage of recombinant ADAMTS13 by plasmin takes place much less rapidly than in a buffered system, but identical C-terminally truncated products are formed first [13]. It was suggested that these fragments were no longer active.

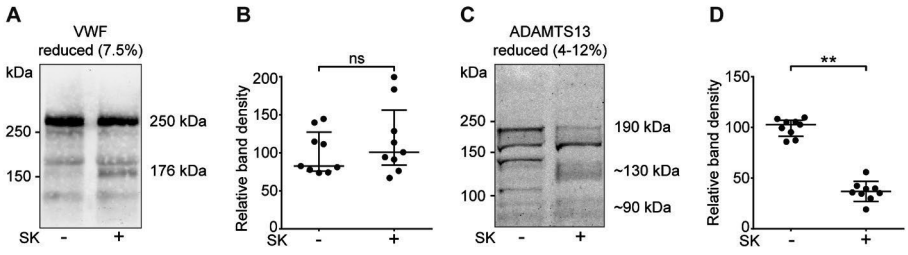


Figure 2. Plasmin predominantly cleaves ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) in plasma under static conditions.

(A-D) The effect of plasmin on von Willebrand Factor (VWF) and ADAMTS13 in plasma was investigated by triggering plasminogen (PLG) activation with streptokinase (SK; 1,000 U/mL, 30 minutes at 37°C), followed by addition of inhibitor cocktail (100 μM PPACK, 250 KIU/mL aprotinin, 100 U/mL hirudin) to terminate plasmin activity. As a control, plasma was incubated with buffer without streptokinase and inhibited with inhibitor cocktail as before. (A, C) Plasma (0.5 μL) was separated per lane by SDS-PAGE and immunoblotted for VWF (A), and ADAMTS13 (C), under reduced conditions. Representative immunoblots are shown. (B,D) Quantification of band densities of multiple experiments (n = 9) presented as median ± interquartile range. Statistical analysis was performed with Wilcoxon matched-pairs signed-rank test (average band density of three control samples per experiment was set to 100%). Results were considered statistically significant when $p \leq 0.05$ (ns, non-significant; ** $p \leq 0.01$).

During our next experiments, we made a striking observation. When plasminogen activation was triggered in plasma, FRETS-VWF73 conversion was increased by 21.6 ± 6.6%, compared with buffer control plasma samples (*P* = 0.0313; Figure 3A). In our previous experiment (Figure 1B), we had found that plasmin does not elicit FRETS-VWF73 substrate fluorescence in a buffered system. This led us to hypothesize that plasmin indirectly contributes to increased FRETS-VWF73 conversion in plasma. To investigate this, we modified the FRETS-VWF73 assay into a two-step procedure. First, plasmin activity was triggered. Second, inhibitor cocktail was added to reduce plasmin activity to less than 1% (Figure 3B). When FRETS-VWF73 conversion was determined in plasma in which plasmin had been active, we consistently detected an increase in substrate conversion compared with buffer control plasma samples (21.2 ± 6.9%, *P* = 0.0313; Figure 3C). This indicates that, although plasmin cleaves ADAMTS13, its apparent activity increases. Next, we set out to identify the responsible mechanism.

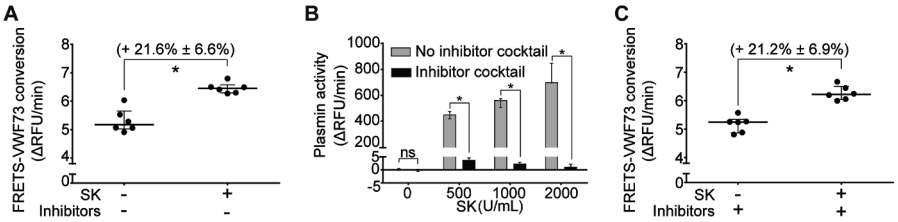


Figure 3: Plasmin enhances ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) activity in plasma.

(A) Activation of plasminogen (PLG) with streptokinase (SK) in plasma (1,000 U/mL, 30 minutes at 37°C) increases ADAMTS13 activity (+21.6 ± 6.6%, *p* = 0.0313) measured in the FRETS-VWF73 assay compared with the buffer control (i.e., without SK). (B) Plasmin activity that is generated in plasma by SK is effectively inhibited by inhibitor cocktail (grey bars = uninhibited buffer control, black bars = inhibitor cocktail). (C) Transient plasmin activity also increases ADAMTS13 activity (+21.2 ± 6.9%, *p* = 0.0313) measured in a modified two-stage FRETS-VWF73 assay. In this assay setup, plasma plasminogen was temporarily activated with streptokinase (buffer without streptokinase was included as a control) and subsequently inhibited with inhibitor cocktail. Panels (A) and (C) represent initial rates of substrate conversion presented as median ± interquartile range, whereas panel (B) is presented as mean ± standard deviation (SD). Statistical analysis was performed with Wilcoxon matched-pairs signed-rank test. All values represent compound data of three separate experiments, performed in duplicate. Results were considered statistically significant when $p \leq 0.05$ (ns, non-significant; *$p \leq 0.05$).

Plasmin generates C-terminally truncated ADAMTS13 products with VWF-binding properties

The interaction between the spacer domain of ADAMTS13 and the A2 domain of (unfolded) VWF is important for efficient VWF cleavage. A recent study elegantly demonstrates that the C-terminal complement C1r/C1s, Uegf, Bmp1 (CUB) domains of ADAMTS13 limit its function by intramolecular binding to the spacer domain [14]. The resulting 'closed'

conformation can be opened by binding to globular VWF, unlocking the full activity of ADAMTS13. Interestingly, this same study, as well as an earlier study [15], showed that recombinant ADAMTS13 variants either lacking the C-terminal tail or the CUB domains displayed an increased activity without initial binding to globular VWF.

In our next experiments, we investigated the effect of plasmin on the VWF-binding properties of ADAMTS13. Hereto, we incubated plasma samples on immobilized VWF in microtiter plates.

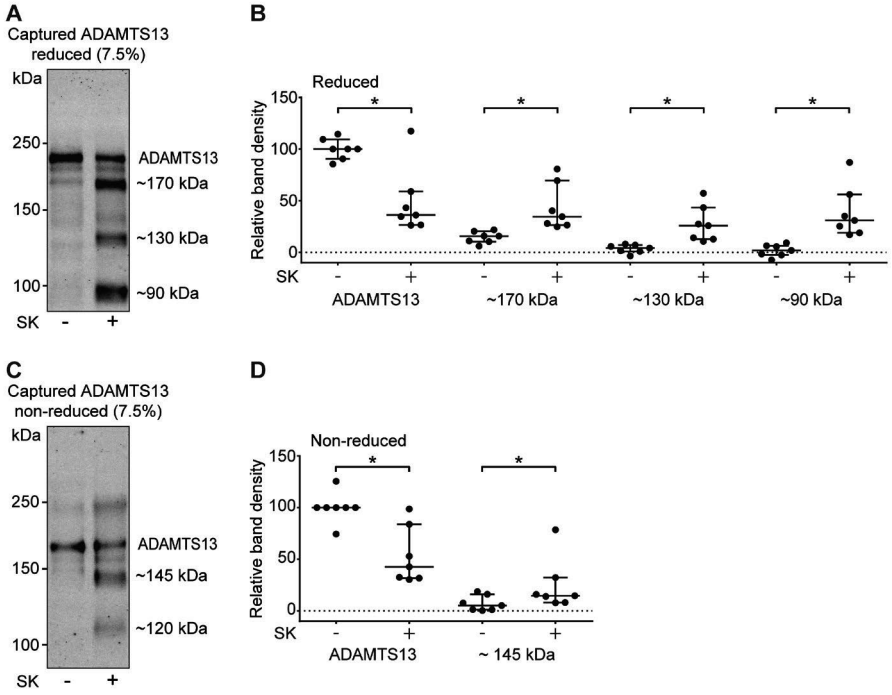


Figure 4: Plasmin generates truncated ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) variants with VWF-binding properties and a metalloprotease domain.

(A-D) The binding of plasma ADAMTS13 to immobilized VWF was analysed by immunoblotting with an antibody against the metalloprotease domain under reducing (A,B) and non-reducing conditions (C,D). Plasmin activity was transiently triggered with the two-stage approach in which plasma plasminogen is activated with streptokinase and subsequently inhibited with inhibitor cocktail prior to the incubation of the plasma samples on immobilized VWF (in presence of ethylenediaminetetraacetic acid [EDTA]). As a control, buffer without streptokinase was included. (A-D) Representative immunoblots and quantification of band densities from repeated experiments (median ± interquartile range, n = 7). Statistical analysis was performed with Wilcoxon matched-pairs signed-rank test. Results were considered statistically significant when $p \leq 0.05$ (*$p \leq 0.05$).

After washing, bound ADAMTS13 was eluted with sample buffer and analysed by western blotting with an antibody against the metalloprotease domain [8]a metalloprotease, cleaves von Willebrand factor (VWF. In plasma, the major product that is captured is 190 kDa (Figure 4A and B; reducing conditions). A second minor product is seen at approximately 170 kDa. When plasmin activity is triggered and terminated in plasma before incubation on immobilized VWF, less full-length protein (190 kDa) binds. In this case, multiple truncated species of approximately 170, 130 and 90 kDa are captured. ADAMTS13 contains intramolecular disulfide linkages; therefore, these fragments could remain covalently associated under non-reducing conditions. Analyses under non-reducing conditions show a highly similar picture: intact ADAMTS13 is captured from plasma before, but much less after exposure to plasmin. Instead, truncated variants that contain the protease domain are captured (Figure 4C and D).

Truncated ADAMTS13 in systemic amyloidosis

Patients with systemic amyloidosis are in a chronic hyperfibrinolytic state, which may contribute to their reduced haemostatic potential and bleeding problems [16]. We investigated ADAMTS13 in these patients and control samples (n = 11 and 8, respectively), of which PAP had been previously determined. In line with our earlier report, PAP complex levels differed significantly between controls (median; 171.5 ng/mL; inter quartile range [IQR] 130.8-221.8 ng/mL) and patients (median; 1,238 ng/mL; IQR 475.8-4,103 ng/mL) (Figure 5A). We next investigated ADAMT13 truncation in these patient plasmas. As before, we analysed ADAMTS13 binding to immobilized VWF. In our earlier experiments (Figure 4A), plasmin generates a 170-kDa C-terminally truncated ADAMTS13. To a minor extent, this product is already present in normal plasma (Figure 4A). We found that this 170-kDa band was represented more strongly in plasma of patients with systemic amyloidosis than of controls (Figure 5B shows a representative blot). To correct for inter-individual differences, we expressed truncated 170-kDa ADAMTS13 relative to 190-kDa ADAMTS13 for each plasma sample. The presence of truncated 170-kDa ADAMTS13 correlates with the extent of fibrinolytic activity (i.e., PAP complexes; r = 0.5491, p = 0.0149). These findings were not dependent on the patient with the highest PAP complex levels and accompanying ADAMTS13 truncation (r = 0.4696, p = 0.0493 after exclusion). ADAMTS13 activity in these samples was determined with the FRETS-VWF73 assay (Figure 5D), but no significant difference was found between the control and patient groups. We observed greater than 20% variation in ADAMTS13 activity between various plasma samples, which is in good correspondence to earlier reports [5]ADAMTS13, cleaves von Willebrand factor (VWF. This may obscure increased activity as a function of plasmin-mediated truncation.

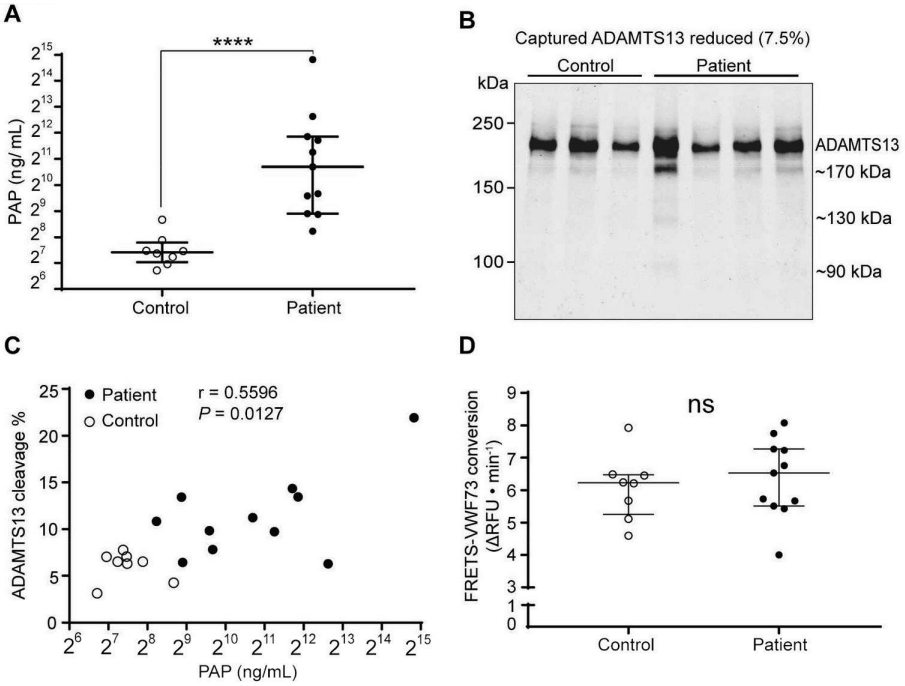


Figure 5. Truncated ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) in systemic amyloidosis.

(A) Plasmin-α2-antiplasmin complex levels (PAP) in systemic amyloidosis patients and controls without systemic amyloidosis (n = 11 and 8, respectively). Plasma samples were incubated on immobilized VWF for ADAMTS13 binding (in presence of EDTA). (B) Bound ADAMTS13 was analyzed by immunoblotting. (C) Correlation plot of PAP complex levels and truncated ADAMTS13 (170/190 kDa ADAMTS13 ratio) of these samples (r = 0.5491, p = 0.0149). (D) ADAMTS13 activity of systemic amyloidosis patients and controls, measured with the FRETS-VWF73 assay. (A,D) Statistical analysis was performed with Wilcoxon matched-pairs signed-rank test. (B) Representative immunoblot. (C) Correlation was tested using non-parametric Spearman correlation. Results were considered statistically significant when $p \leq 0.05$ (ns, non-significant; ****$p \leq 0.0001$).

Discussion

We here report the finding that plasmin under static conditions in plasma rapidly truncates ADAMTS13 and this is accompanied by increased ADAMTS13 activity in the FRETS-VWF73 assay. Moreover, multiple truncated forms of ADAMTS13 retain the capacity to bind to VWF. In our study, we ruled out that plasmin is responsible for the observed increased FRETS-VWF73 substrate fluorescence. In contrast, we found that plasmin can destroy FRETS-VWF73 substrate in a buffered system. Although this effect is modest in normal plasma that contains the powerful plasmin inhibitor α2-antiplasmin, it might generate

an analytic threat in plasma samples with partial [2] or complete [13] α2-antiplasmin deficiency. If needed, this potential analytical threat can be averted with the use of plasmin-blocking serine protease inhibitors, as we used in this study.

Plasmin can cleave ADAMTS13 in buffer at multiple positions: at Arg-257/Ala-258 (metalloprotease domain), at Arg-888/Thr-889 (linker region between the fourth and fifth thrombospondin type 1 repeat [TSP-1]) and at Arg-1176/Arg-1177 [3,17]. It is likely that similar cleavage events take place during plasminogen activation in plasma, corresponding to the truncated ADAMTS13 products that can bind to immobilized VWF (Figure 4A and B). Intramolecular disulphide-linkage assures covalent association of the fragments generated by cleavage at Arg-257/Ala258 or Arg-888/Thr-889 [3,18]ADAMTS13. It is unclear how ADAMTS13 activity is regulated within the vascular system. In the absence of a regulatory mechanism, ADAMTS13 activity might compromise platelet adhesion at sites of vascular injury. We hypothesized that at sites of vascular injury, ADAMTS13 activity could be regulated locally by coagulation proteinases. Initiation of coagulation in human plasma resulted in the disappearance of added full-length recombinant ADAMTS13. This loss was inhibited by hirudin. Using purified proteins, we showed that ADAMTS13 is proteolyzed at several cleavage sites by thrombin in a time- and concentration-dependent manner. Furthermore, this proteolysis ablated ADAMTS13 activity against purified von Willebrand factor. Preincubation of thrombin with soluble thrombomodulin, but not heparin, inhibited the proteolysis of ADAMTS13, suggesting the involvement of thrombin exosite I (and not exosite II. However, C-terminal truncation after cleavage at Arg-1176/Arg-1177 truncates ADAMTS13 by removal of the eighth TSP-1 and both CUB domains [17,18]thrombotic thrombocytopenic purpura. We established a simple and efficient method to purify plasma ADAMTS13 (pADAMTS13. This is consistent with the capture of truncated ADAMTS13 under non-reduced conditions (Figure 4C and D).

Based on our findings, we proposed that monomeric ADAMTS13 is C-terminally truncated by plasmin. However, it is noteworthy that we observed a second plasmin-sensitive ADAMTS13-immunoreactive high-molecular weight product (~350 kDa) product in plasma (Supplementary Figure S3). Future studies will have to determine whether this species of ADAMTS13 contributes to the observed increased ADAMTS13 activity after plasminogen activation.

When ADAMTS13 is not 'in action', its CUB domains interact with its spacer region to ensure a closed conformation. During VWF binding, these CUB domains interact with VWF [19]. This causes ADAMTS13 to change conformation, which increases its activity ('conformational activation') [14]. We hypothesize that plasmin-mediated removal of CUB domains causes ADAMTS13 to adopt an open conformation (resembling conformational activation [14]) with increased accessibility of the spacer domain for unfolded VWF.

We propose that our findings reflect a physiological mechanism to enhance ADAMTS13

activity. When the microvasculature senses hypoxia, as may occur during microthrombosis, plasmin is locally generated on endothelial cells. Besides its direct effects on VWF [4,20]176, and 140 kD fragments cleaved from the 225 kD subunit. A monoclonal antibody map of vWF, based on the reactivity of individual antibodies with cyanogen bromide and tryptic fragments of known carboxy and/or amino termini, showed that in normal and IIA von Willebrand disease (vWD and platelet-VWF complexes [2], plasmin cleaves ADAMTS13, leading to enhanced activity. It is attractive to hypothesize that similar truncation of ADAMTS13 and associated increased activity contributes to the haemorrhagic phenotype in hyperfibrinolytic disorders, such as systemic amyloidosis.

Conclusions

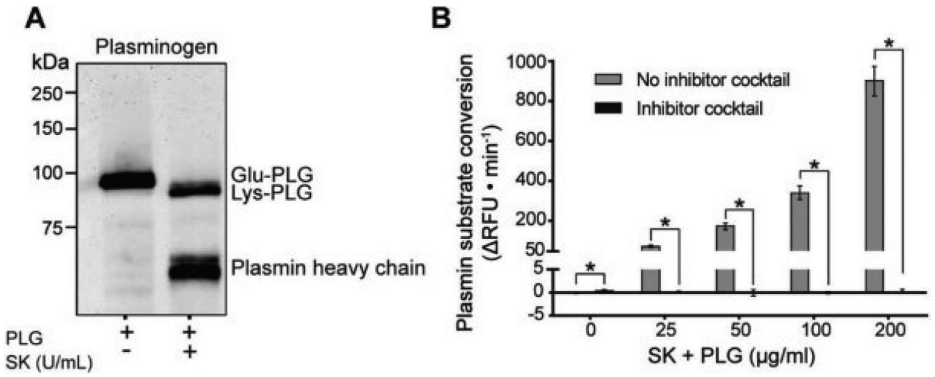
Our findings indicate that under static conditions in plasma, plasmin cleaves ADAMTS13 more rapidly than VWF. C-terminally truncated ADAMTS13 can bind to immobilized VWF and is associated with an enhanced activity. Matching C-terminally truncated ADAMTS13 forms are generated *in vivo* during increased fibrinolytic activity.

Supplementary data to Clark *et al.* 'Truncation of ADAMTS13 by plasmin enhances its activity in plasma.'

Supplementary results

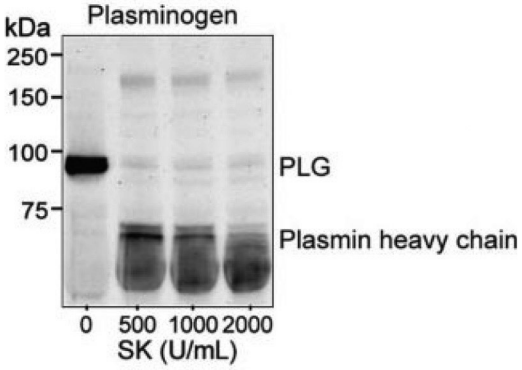
Plasmin releases ADAMTS13 from a disulfide-linked complex.

Under non-reducing conditions, we observed a ~350 kDa ADAMTS13-immunoreactive band in plasma that almost completely disappears after exposure to plasmin (-91.0%±10.1%, p = 0.0039, Supplementary Figure S3A and B). It is noteworthy this product normally does not bind to immobilized VWF (Figure 4C; upper part of the immunoblot) and is not seen in the presence of DTT (i.e., the product is disulfide-linked). Interestingly, plasma also contains a VWF-immunoreactive product that migrates at an identical height (~350 kDa) and is modestly consumed by plasmin (- 23.1% ± 13.1%, p = 0.0078, Supplementary Figure S3C and D). This latter product is proposed to represent a disulfide-linked homodimer of C-terminally truncated VWF molecules [1-3]. More precise electrophoretic separation revealed that the ~350 kDa product actually consists of multiple bands. The lower is immunoreactive for both VWF and ADAMTS13 prior to, but not after plasmin activity (Supplementary Figure S3E). This suggests that a fraction of ADAMTS-13 circulates in disulfide-linked high molecular weight complexes [1,4-6].



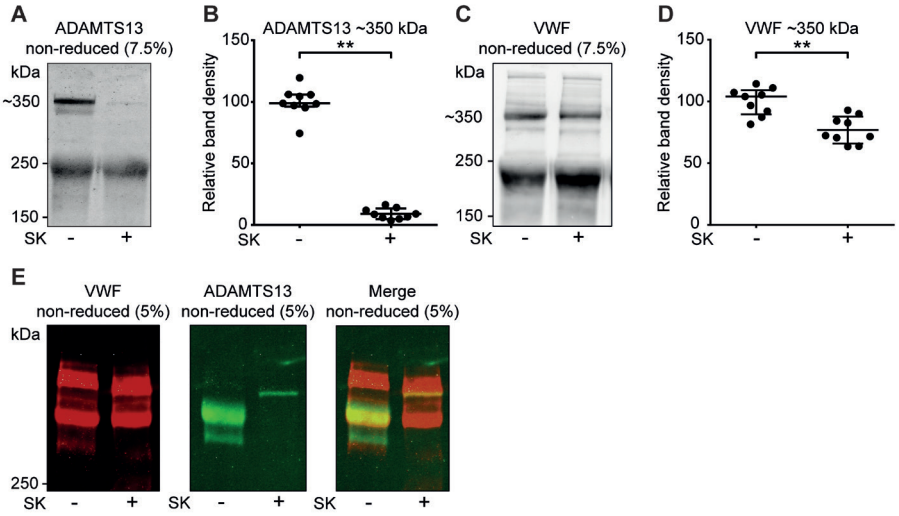
Supplementary Figure S1. Activation of purified plasminogen with streptokinase.

(A) Representative immunoblot of purified plasminogen (PLG) after incubation with 50 U/mL streptokinase (SK) or vehicle for 30 minutes at 37 °C. 100 ng per lane of purified plasminogen was separated on 4-12% gradient gel under reducing conditions and immunoblotted for plasminogen. Activation of plasminogen with streptokinase results in conversion of Glu-plasminogen (86 kDa) into Lys-plasminogen (83 kDa) and formation of two-chain plasmin, indicated by appearance of the plasmin heavy chain (60 kDa). (B) Streptokinase-triggered plasmin activity and inhibition with inhibitor cocktail. Streptokinase-activated plasminogen (25-200 µg/mL in HBS) was incubated with 12.5 µM plasmin substrate I-1390 in the absence or presence of inhibitor cocktail. Bars represent initial rates of fluorescence as mean ± SD of three separate experiments, performed in duplicate. Statistical analysis was performed with Friedman test with Dunn's multiple comparisons test where all values are compared to 0 µg/mL activated plasminogen.



Supplementary Figure S2. Activation of plasma plasminogen with streptokinase.

(A) Representative immunoblot of plasma plasminogen (PLG) that was activated with different concentrations of streptokinase (SK) and inhibited with inhibitor cocktail. 0.25 µL of reduced plasma sample was loaded per lane. Activity assays are shown in Figure 3B.



Supplementary Figure S3. Plasmin liberates ADAMTS13 from disulfide-linked complexes.

(A and C) Immunoblotting of plasma under non-reducing conditions revealed a high molecular-weight product, which is immunoreactive for both ADAMTS13 and VWF, respectively. (B and D) This product is susceptible to plasmin activity. (E) Detailed electrophoretic separation and immunoblotting for ADAMTS13 and VWF revealed that one of these bands is immunoreactive for both proteins. Panels A-D represent representative immunoblots and quantification of bands densities (median ± interquartile range) are shown. Statistical analysis was performed with Wilcoxon matched-pairs signed rank test (average band density of three control samples on the same blot was set to 100%). Results were considered statistically significant when $P \leq 0.05$ (** indicates $P \leq 0.01$).

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6

CHAPTER 6

Tissue-type plasminogen activator binds to many *Schistosoma mansoni* proteins and enhances plasminogen activation

Mirjam M. Mebius[1,2], Jody M. J. Op Heij[2], J. Mirjam A. Damen[3], Renske A. van Gestel[3], Aloysius G. M. Tielens[1,4], Philip G. de Groot[2], Rolf T. Urbanus[2], Jaap J. van Hellemond[1]

[1] Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center Rotterdam, Rotterdam, the Netherlands
[2] Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[3] Biomolecular Mass Spectrometry & Proteomics, Utrecht Institute for Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, the Netherlands
[4] Department of Biochemistry and Cell Biology, Faculty Veterinary Medicine, Utrecht University, the Netherlands

Abstract

Plasminogen binding to the outer surface of pathogens is a well-known virulence factor for many pathogens, facilitating invasion, dissemination, and evasion of the immune system and blood coagulation system. Plasminogen binding has been studied extensively in parasitic worms belonging to the *Schistosoma* genus, leading to the identification of several proteins that bind and activate plasminogen. In *S. mansoni,* plasminogen activation at the outer surface occurs exclusively in the presence of tissue-type plasminogen activator (tPA). This led us to investigate the schistosomal proteins involved in tPA binding at the outer surface of the parasite. Using ligand blotting and pull-down experiments, numerous tPA binding proteins were identified. This indicates that *S. mansoni* contains many proteins that can interact with tPA and could be involved in localization of tPA to the outer surface of the parasite, which can facilitate the acceleration of activation of plasminogen to plasmin.

Interaction with the mammalian fibrinolytic system, of which its main enzyme plasmin is involved in degradation of fibrin and extracellular matrix proteins, has been described for a broad range of pathogens, including several parasitic worms. Plasminogen-pathogen interactions facilitate tissue invasion, dissemination, immune evasion, and evasion of blood coagulation [1]. Blood-dwelling parasitic worms belonging to the *Schistosoma* genus, such as *Schistosoma bovis, S. japonicum,* and *S. mansoni*, have also been described to interact with the fibrinolytic system of their mammalian host. The binding and activation of plasminogen at the outer surface, or tegument, of these species, has been extensively studied [2–6] and revealed several plasminogen-binding proteins. The first of these studies was performed in *S. bovis* and showed plasminogen binding by up to ten plasminogen-binding proteins present on the outer surface [2] (reviewed by de la Torre-Escudero *et al.*) [7]). Plasminogen-binding proteins identified were enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and fructose-bisphosphate aldolase (FBA), of which FBA was shown not to be associated with the *S. bovis* tegument and was therefore ruled out as a putative physiological receptor for plasminogen. Additionally, an *S. bovis* annexin has been identified in the worm tegument that was capable of binding plasminogen [5]. In addition to plasminogen-binding, the conversion of plasminogen to plasmin is also enhanced by tegument extracts, both in the presence and absence of the natural activator of plasminogen, tissue-type plasminogen activator (tPA) [2]. Of the plasminogen-binding proteins, only enolase and annexin were able to activate plasminogen to plasmin and this conversion was dependent on the presence of tPA [3,5]. Later, enolase proteins were also identified on the surface of *S. japonicum* and *S. mansoni* species [4,6] and were both shown to be involved in plasminogen-binding. Enhancement of plasminogen conversion to plasmin has only been found for the *S. mansoni* enolase and was also dependent on the presence of tPA [6].

Pathogens that use the fibrinolytic system to their advantage, have been described to bind not only plasminogen to their surface, but often also tPA [1]. However, to date, no studies have been performed on the interaction between *Schistosoma* species and tPA. This study, therefore, focusses on the interaction between *S. mansoni* and tPA to further elucidate how *S. mansoni* interacts with the fibrinolytic system of their host.

We first examined the conversion of plasminogen to plasmin by determining cleavage of a fluorogenic plasmin substrate by *S. mansoni* worm homogenate (WH) (Fig. 1A). This showed that plasminogen activation by *S. mansoni* WH occurs exclusively in the presence of tPA, which is in line with the finding of Figueiredo *et al.* [6]. Similarly, intact live male worms were able to convert plasminogen to plasmin in a dose-dependent manner (Fig. 1B), again, exclusively in the presence of tPA (not shown). This finding of tPA-dependent plasminogen activation on intact male worms indicates that the plasminogen activating molecules of *S. mansoni* are located on the outer surface

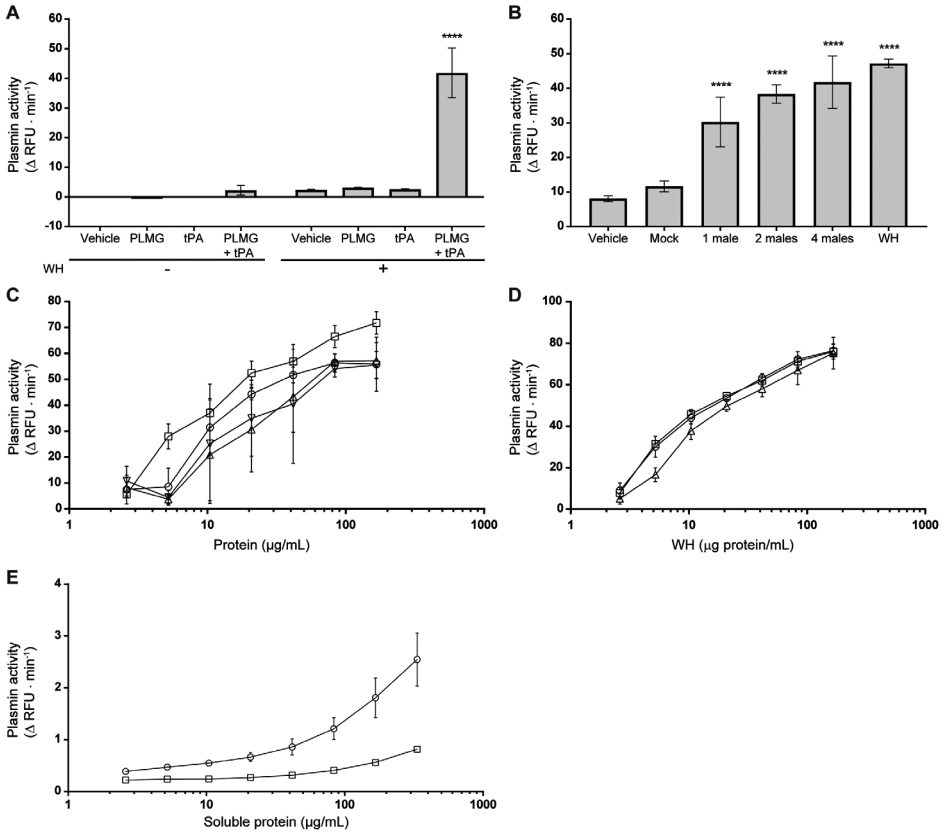

Figure 1. *S. mansoni* enhances plasminogen activation in a tPA dependent manner.

A) Plasmin activity detected by cleavage of the plasmin substrate (Boc-Val-Leu-Lys-AMC, Bachem, Bubendorf, Switzerland) (33 µM) in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) in the presence or absence of 333 µg/mL *S. mansoni* worm homogenate (WH). WH was prepared in HBS by homogenization (10% v/v) of intact adult *S. mansoni* pairs isolated from Golden hamsters in HBS followed by centrifugation at 500x g for 5 minutes at 4 °C. Where indicated 13 µg/mL plasminogen (purified from plasma as described by de Maat *et al.* [14]) and/or 0.1 µg/mL tPA (Actilyse, alteplase, Boehringer Ingelheim, Ingelheim, Germany) was added. Fluorescence was monitored for 2 hours at 37°C on a Spectromax M^2 fluorescence microplate reader (MTX Lab Systems, Bradenton, FL, USA) equipped with a 390 nm excitation filter and 460 nm emission filter. B) Plasmin activity detected by cleavage of the plasmin substrate (33 µM) in HBS supplemented with plasminogen (13 µg/mL) and tPA (0.1 µg/mL) in the presence or absence of live *S. mansoni* males or 167 µg/mL WH. C) Plasmin activity detected by cleavage of the plasmin substrate (33 µM) in HBS in the presence of tPA (0. 1 µg/mL) and 2.6 µg/mL - 167 µg/mL of different fractions of *S. mansoni*: WH (o), *S. mansoni* soluble protein fraction (□] (obtained by 14000g centrifugation of WH for 30 minutes at 4°C), tegumental membrane fraction (Δ), and total membrane fraction (Ñ). Both membrane fractions were isolated by a freeze–thaw method according to Roberts *et al.* [15] as described by Brouwers *et al.* [16]. D) Plasmin activity detected by cleavage of the plasmin substrate (33 µM) in HBS supplemented with plasminogen (13 µg/mL) and tPA (0.1 µg/mL) and 2.6 µg/mL - 167 µg/mL WH in the presence or absence (o) of 5 mM EDTA (□) and 5 mM $CaCl^{2+}$ (Δ). E) Plasmin activity generated by 2.6 µg/mL - 333 µg/mL WH in 25% normal platelet poor plasma (Sanquin, Amsterdam, the Netherlands) in the presence (o) or absence (□) of tPA (0.1 µg/mL) as detected by cleavage of the plasmin substrate (33 µM). For all experiments, initial rates of fluorescence are represented as mean ± SD. All values represent three

replicates of duplo measurements. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Significant differences from control conditions (reagents themselves without parasites) were calculated using a one-way analysis of variance (ANOVA) and Bonferroni as the post hoc test. *p*-values were considered significant at $p \leq 0.05$ (**** indicates $p \leq 0.0001$).

of the parasite and is in line with the findings of Fiqueiredo *et al.* [6]. In contrast, the conversion of plasminogen to plasmin by *S. bovis* tegument extracts has been described to occur in the absence of tPA as well [2], indicating the possibility of species differences in the mechanisms used for plasminogen activation.

To further elucidate the *S. mansoni* molecules that could be involved in the interaction with the fibrinolytic system, we examined to what extent different extracts of *S. mansoni* are able to convert plasminogen to plasmin (Fig. 1C). Four different extracts were examined: WH, a soluble protein fraction, a tegumental membrane fraction and a total membrane fraction. Plasminogen activating activity was found in all examined fractions of the worm. The profound differences in protein composition of these fractions indicate that it is unlikely that a single *S. mansoni* protein is responsible for the observed conversion of plasminogen to plasmin. This hypothesis is further strengthened by the fact that suppression of enolase expression by RNAi described by Figueiredo *et al.* did not affect the ability of the worms to activate plasminogen, while *S. mansoni* enolase is known to enhance the conversion of plasminogen to plasmin [6]. Furthermore, addition of a chelating agent (EDTA) or an excess of Ca^{2+}-ions did not significantly alter the conversion of plasminogen to plasmin by *S. mansoni* WH (Fig. 1D), indicating that the observed activity is most likely not mediated by the known *S. mansoni* plasminogen-binding protein, enolase [6], or a homologue to the annexin protein described for *S. bovis* [5], as both their activity is dependent on bivalent metal ions. However, it is not known whether the catalytic activity, dependent on bivalent metal ions, is necessary for the role of enolase and annexin in the conversion of plasminogen to plasmin.

Next, we examined whether the conversion of plasminogen to plasmin by *S. mansoni* also occurs in a plasma environment, as all plasminogen activation experiments performed to date have been done with purified plasminogen in a buffer environment. As shown in Fig. 1E, the *S. mansoni* soluble protein fraction can activate plasminogen in plasma. The activation of plasminogen by *S. mansoni* soluble proteins is strongly enhanced by the addition of tPA. However, some activation of plasma plasminogen is observed without addition of tPA, which suggests some tPA-independent conversion of plasminogen to plasmin at high concentrations of *S. mansoni* soluble proteins.

The strict tPA-dependence of *S. mansoni* plasminogen converting activity, led us to the hypothesis that tPA may be bound by components at the outer surface of *S. mansoni,* similar to what was described for plasminogen. Therefore, we examined the presence of tPA binding proteins in adult *S. mansoni*. An *S. mansoni* soluble protein fraction was separated by SDS-PAGE and tPA binding proteins were visualized with subsequent

ligand blotting (Fig. 2A). This revealed the presence of numerous tPA binding proteins with sizes varying from 15 kDa to 250 kDa. Although tPA is also known to bind to partially denatured proteins through specific recognition of cross-β structures by the tPA finger domain [8], it is unlikely that this is the reason why so many tPA binding proteins in the ligand blot are observed. Clear bands are detected instead of a smear and there are clear differences between the bands observed in the SDS-PAGE and the ligand blot. This suggests the recognition of specific (linear) epitopes of *S. mansoni* proteins by tPA. Further experiments demonstrated that the interactions between tPA and *S. mansoni* proteins are not affected by the addition of the lysine-analog ε-aminocaproic acid (ε-ACA) as demonstrated by ligand blotting (Fig. 2B), suggesting that the interaction between tPA and *S. mansoni* proteins is lysine-independent.

Next, a pull-down assay with tPA was performed on the *S. mansoni* soluble protein fraction (Fig. 2C). The aim of this experiment was to identify tPA binding proteins exposed on the outer surface of the worm. A limitation of this pull-down assay is that it can only be performed with solubilized or soluble proteins, and thus a tegumental membrane fraction cannot be used in this assay. However, there is considerable overlap between proteins present in the soluble protein fraction and in the *S. mansoni* tegument, which is why we choose to analyze the *S. mansoni* soluble protein fraction in the pull-down assay. The pull-down assay was performed with biotin-labeled tPA (tPA-PPACK-biotin), in which tPA is inhibited in its active site by biotin-labeled Phe-Pro-Arg-Chloromethyl ketone (PPACK-biotin). In interaction studies, the use of this indirect labeling strategy is advantageous over the more commonly used amine-coupling based strategy, as labeling with PPACK-biotin does not affect binding sites on relevant domains of tPA. After separation of the proteins with SDS-PAGE, three bands were observed that clearly differed between the pull-down with and without tPA-PPACK-biotin at ~100 kDa, ~70 kDa, and ~30 kDa, respectively. The protein band with a molecular weight of ~70 kDa that was observed only in the tPA-PPACK-biotin positive condition, most likely consists of the tPA that has been used in the pull-down, as tPA has a molecular weight of 68 kDa. In order to identify tPA binding proteins from the pull-down assay, lanes were divided into 12 bands of approximately equal size and proteins were subjected to in-gel tryptic digestion as described by Shevchenko *et al.* [9] and analyzed with LC-MS/MS. Analysis of the data was performed with Proteome Discoverer using the Swiss-Prot database limited to Rodentia as taxonomy, to exclude any contaminating host (Golden hamster) proteins from the analysis, and the S. mansoni database (Schistosoma_mansoni_v5.2.fa, Wellcome Trust Sanger Institute, Hinxton, UK, accessible from ftp://ftp.sanger.ac.uk/pub/pathogens/Schistosoma/mansoni/Latest_assembly_annotation_others/) for annotation of the peptides. Tegument localization of the identified proteins was based upon a literature search for tegumental proteome studies performed for *S. mansoni* [10–12]. LC-MS/MS analysis

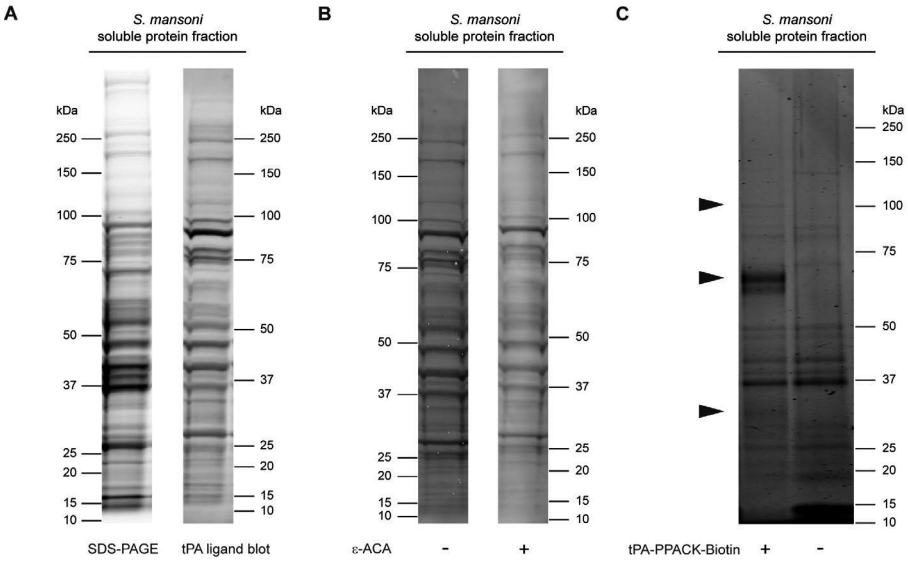

Figure 2. tPA Ligand blotting and pull-down experiments reveal tPA binding proteins of S. mansoni.

A) SDS-PAGE and tPA ligand blot of *S. mansoni* soluble protein fraction. SDS-PAGE was performed with 30 µg *S. mansoni* soluble protein fraction per lane in 3x sample buffer (187 mM Tris-Cl (pH 6.8), 30% (v/v) glycerol, 6% SDS, 0.003% Bromophenol blue) on a 10-wells Bolt™ 4-12% Bis-Tris Plus Gel (ThermoFischer Scientific, Waltham, MA, USA). SDS-PAGE was performed in Bolt™ MOPS SDS Running Buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 45 minutes at 165V. Protein bands were visualized with PageBlue™ Protein Staining Solution (Thermo Fisher Scientific, Waltham, MA, USA) for 1h at RT. For ligand blotting, SDS-PAGE separated proteins were transferred onto an Immobilon®-FL PVDF membrane (Merck, Darmstadt, Germany) at 125V for 60 minutes in blotting buffer (25 mM Tris-HCl, 192 mM glycine, 20% ethanol) and blocked with blocking buffer (0.5x Odyssey® Blocking Buffer (TBS) (LI-COR Biosciences, Lincoln, NE, USA)) for 1 hour at RT. Blots were washed with TBS-0.05% Tween20 (TBST) and incubated with 50 ug/mL tPA in blocking buffer overnight at 4°C. Blots were washed with TBST and bound tPA was detected by incubation with a monoclonal mouse anti-human tPA antibody (ab21049, Abcam, Cambridge, UK) in blocking buffer (1:3,000) for 2 hours at RT. Blots were washed with TBST and the primary antibody was detected with IRDye 800 donkey anti-mouse IgG in blocking buffer (1:5000) for 2 hours at RT. Blots were washed with TBST and analyzed on a near-infrared Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA). B) Ligand blots of *S. mansoni* soluble protein fraction (30 µg/lane) with or without the addition of 25 mM ε-aminocaproic acid (ε-ACA) during the tPA incubation step. C) Pull-down experiment of *S. mansoni* soluble protein fraction with PPACK-biotin-labelled tPA. tPA (2 mg/mL in water) was incubated with PPACK-biotin (339 µM) (Biotinylated FPR-chloromethylketone, BFPRCK-06, Haematologic Technologies Inc., Essex Junction, VT, USA) for 1 hour at 37°C, followed by purification of the PPAC-biotin-labelled tPA with a 5 mL Zeba Spin Desalting Columns (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. *S. mansoni* soluble protein fraction (1.6 mg/mL) was incubated with or without PPACK-biotin-labelled tPA (100 µg/mL) for 1 hour at RT, followed by 30 minutes incubation at RT with 4.6% Pierce™ streptavidin agarose (Thermo Fisher Scientific, Waltham, MA, USA). Beads were washed twice with HBS, transferred in 3x sample buffer and boiled for 10 minutes at 100 °C. Samples were separated with SDS-PAGE and protein bands were visualized with PageBlue™ Protein Staining Solution as described above. Protein bands that are exclusively visualized in the pull-down with tPA-PPACK-biotin are indicated by black arrowheads.

revealed the presence of at least 10 tegumental proteins that were exclusively present in the tPA-PPACK-biotin positive lane (Supplemental Table 1A). Additionally, 24 tegumental proteins were found that were enriched in the tPA-PPACK-biotin positive lane, but also present in the control lane (Supplemental Table 1B). Supplemental table 1C and 1D show the non-tegumental proteins that were exclusively present (35) or enriched (30) in the tPA-PPACK-biotin positive lane compared to the tPA-PPACK-biotin negative lane, respectively. Amongst the tegumental proteins that were enriched or exclusively present in the tPA-PPACK-biotin positive lane, several classical tegument proteins were identified, including glutathione S-transferase, GAPDH, and leucine aminopeptidase [13]. Interestingly, the tegumental proteins that are identified exclusively in the tPA-PPACK-biotin positive lane have very diverse functions, including signal transduction, metabolic functions, cytoskeletal organization, stress response and chaperones, and peptidase activities. This would indicate that proteins with diverse functions have an affinity for tPA, which would explain why so many proteins were found to bind tPA. Several identified proteins that were enriched in the tPA-PPACK-biotin positive lane were previously identified as plasminogen-binding proteins, such as GAPDH, FBA, and enolase, suggesting a potential double role for these proteins in the interaction with the fibrinolytic system. However, some of the identified tPA binding proteins in the tegument may not be exposed on the outside of the worm, as has been described for FBA [7], and therefore these proteins may not be available for binding of tPA.

Of the 10 tegumental proteins exclusively present in the tPA-PPACK-biotin positive lane, major vault protein and chaperonin containing TCP1 are the most abundant proteins, and these proteins are involved in signal transduction and protein folding, respectively. Of the 24 tegumental proteins that were found to be enriched in the tPA-PPACK-biotin positive lane, tubulin and GAPDH were the most abundant proteins. Tubulin is a cytoskeletal protein, while GAPDH is a metabolic protein and has additionally been demonstrated to be able to bind plasminogen and enhance its conversion to plasmin. However, the most enriched proteins are cathepsin B, a gut cysteine peptidase, a heat shock 70 kDa protein, involved in stress responses, and calpain, a calcium-dependent cysteine peptidase.

Of the non-tegumental proteins exclusively present in the tPA-PPACK-biotin positive lane glutamyl tRNA synthetase is the most abundant protein identified, while for the non-tegumental proteins enriched in the tPA-PPACK-biotin positive lane again tubulin and actin proteins are the most abundant.

Further investigation is required to identify the *S. mansoni* proteins that could interact with tPA on the outer surface of the parasite. Nonetheless, the large number of tegumental proteins identified as enriched or exclusively present in the tPA-PPACK-biotin positive lane indicates that *S. mansoni* tegument contains many proteins that can interact with tPA and could be involved in binding of tPA to the outer surface of the parasite, allowing acceleration of activation of plasminogen to plasmin.

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Supplementary data to Mebius *et al.* 'Tissue-type plasminogen activator binds to many *Schistosoma mansoni* proteins and enhances plasminogen activation.'

Supplementary methods

LC-MS/MS analysis of tPA-PPACK-biotin pull-down and control samples

After digestion samples were analyzed on an Orbitrap Q-Exactive (Thermo Fisher Scientific, Waltham, MA, USA) connected to a UHPLC Proxeon Easy-nLC 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were trapped on a double-fritted trap column (Dr. Maisch Reprosil C18, 3 µm, 2 cm × 100 µm (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany)) and separated on an analytical column (Agilent Zorbax SB-C18, 1.8 µm, 40 cm × 75 µm (Agilent, Santa Clara, CA, USA)). Solvent A consisted of 0.1 M acetic acid, solvent B of 0.1 M acetic acid in 80% acetonitrile. Samples were loaded at a pressure of 800 bar with 100% solvent A. Peptides were separated by a 30 min gradient of 10–37% buffer B followed by 30–100% B in 2 min, 100% B for 2.5 min at a flow rate of 150 nL/min. Full scan MS spectra were acquired in the Orbitrap (350-1500 m/z, resolution 35,000, AGC target 3e6, maximum injection time 250 ms). The 20 most intense precursors were selected for HCD fragmentation (isolation window 1.2 Da, resolution 17,500, AGC target 5e4, maximum injection time 120 ms, first m/z 100, NCE 33%, dynamic exclusion 60 s). The results were filtered using Percolator (Käll *et al.*[1] and Spivak *et al.*[2]) to a false discovery rate (FDR) below 1%. We further only accepted peptides with at least six amino acid residues, a Mascot ion score of at least 20, and search engine rank of 1 and at least 2 identified peptides for protein identification. Scores (Mascot ion score) and peptide spectrum matches (PSM; total number of identified peptide spectra matched for the protein) were used to compare the samples. Only proteins that showed a minimum PSM score of 4 and at least a two times enrichment in PSM were considered as enriched proteins.

Supplementary Table 1A: Tegumental protein identifications present exclusively in the tPA-PPACK-biotin pull-down fraction and absent in the control fraction.

		Score		PSM		
Accession number	**Product**	**Control lane**	**Positive lane**	**Control lane**	**Positive lane**	**Localization**
Smp_006740.1	Major vault protein	-	718	-	35	Tegument and non-tegument[a]
Smp_149900.1	Chaperonin containing TCP1, subunit 7 (eta)	-	496	-	40	Tegument and non-tegument[a]
Smp_143150.1	Elongation factor 2	-	334	-	22	Tegument and non-tegument[a]
Smp_037230.1	Fimbrin	-	207	-	9	Tegument[b]
Smp_075790.1	Hemoglobinase (C13 family)	-	178	-	10	Tegument[c]
Smp_030000.1	Leucine aminopeptidase (M17 family)	-	172	-	12	Tegument and non-tegument[a]
Smp_054160.1	Glutathione S-transferase class-mu 28 kDa isozyme	-	172	-	11	Tegument and non-tegument[a]
Smp_059790.1	Transketolase	-	150	-	7	Tegument and non-tegument[a]
Smp_004600.1	T complex protein 1 subunit delta	-	136	-	8	Tegument[d]
Smp_050390.1	Aldehyde dehydrogenase	-	57	-	6	Tegument and non-tegument[a]

[a] Proteins reproducibly identified by van Balkom *et al.* in both the tegumental and non-tegumental fraction of *S. mansoni* (van Balkom *et al.*, supplemental table 1c [3])

[b] Proteins reproducibly and exclusively identified by van Balkom *et al.* in the tegumental fraction of *S. mansoni* (van Balkom *et al.*, supplemental table 1a [3])

[c] Tegumental proteins identified through enzymatic shaving of the tegument surface of *S. mansoni* by Castro-Borges *et al.* (Castro-Borges *et al.*, table 1 [4]).

[d] Tegumental proteins of *S. mansoni* identified by Braschi *et al.* (Braschi *et al.*, table 1 [5]).

Supplementary Table 1B: Tegumental protein identifications present in both pull-down fractions, but enriched in the tPA-PPACK-biotin fraction compared to the control fraction.

		Score			PSM			
Accession number	**Product**	**Control lane**	**Positive lane**	**Ratio**	**Control lane**	**Positive lane**	**Ratio**	**Localization**
Smp_078040.1	Tubulin subunit beta	5906	15031	2.5	232	501	2.2	Tegument and non-tegument[a]
Smp_056970.1	Glyceraldehyde 3 phosphate dehydrogenase	5238	10924	2.1	216	427	2.0	Tegument and non-tegument[a]
Smp_043670.1	ATP-dependent 6-phosphofructokinase	338	2105	6.2	15	83	5.5	Tegument and non-tegument[a]
Smp_041740.1	T complex protein 1 subunit gamma	76	740	9.8	4	49	12.3	Tegument[c]
Smp_042160.2	0-bisphosphate aldolase	227	734	3.2	6	21	3.5	Tegument and non-tegument[e]
Smp_030300.2	Endoplasmin	249	676	2.7	8	26	3.3	Tegument and non-tegument[a]
Smp_017360.1	T complex protein 1 subunit alpha	55	630	11.5	3	38	12.7	Tegument[c]
Smp_099870.1	Eukaryotic translation elongation factor 1 alpha	350	623	1.8	14	31	2.2	Tegument and non-tegument[a]
Smp_103610.1	Cathepsin B-like cysteine proteinase	84	612	7.3	6	35	5.8	Tegument and non-tegument[a]
Smp_049270.1	Major egg antigen (p40)	174	580	3.3	11	26	2.4	Tegument[b]
Smp_038950.1	Lactate dehydrogenase protein	57	536	9.3	5	23	4.6	Tegument and non-tegument[a]
Smp_148530.1	Major egg antigen	100	398	4.0	6	15	2.5	Tegument[c]
Smp_004990.1	T complex protein 1 subunit zeta	83	390	4.7	8	23	2.9	Tegument[c]
Smp_106930.1	Heat shock 70 kDa protein homolog	31	374	11.9	1	22	22.0	Tegument and non-tegument[a]
Smp_065610.2	Pyruvate kinase isozymes M1:M2	72	373	5.2	2	20	10.0	Tegument and non-tegument[a]
Smp_054340.1	Chaperonin containing t complex protein 1 beta	96	359	3.8	15	34	2.3	Tegument[c]
Smp_214190.1	Calpain	26	348	13.5	1	21	21.0	Tegument and non-tegument[a]
Smp_067060.1	Cathepsin B-like cysteine proteinase	22	316	14.5	1	23	23.0	Tegument and non-tegument[e]
Smp_024110.1	Enolase	65	249	3.8	2	15	7.5	Tegument and non-tegument[a]
Smp_043120.1	Universal stress protein	129	233	1.8	3	10	3.3	Tegument and non-tegument[a]
Smp_214060.1	Phosphoglycerate kinase	39	159	4.1	1	7	7.0	Tegument and non-tegument[a]
Smp_186020.1	Major egg antigen	74	144	1.9	6	13	2.2	Tegument[b]
Smp_027610.1	Ribosomal protein S3	98	110	1.1	8	7	2.0	Tegument and non-tegument[e]
Smp_079430.1	GTP binding nuclear protein Ran	45	80	1.8	2	8	4.0	Tegument[d]

[a] Proteins reproducibly identified by van Balkom *et al.* in both the tegumental and non-tegumental fraction of *S. mansoni* (van Balkom *et al.*, supplemental table 1c [3])

[b] Proteins reproducibly and exclusively identified by van Balkom *et al.* in the tegumental fraction of *S. mansoni* (van Balkom *et al.*, supplemental table 1a [3])

[c] Tegumental proteins of *S. mansoni* identified by Braschi *et al.* (Braschi *et al.*, table 1 [5]).

[d] Proteins exclusively identified by van Balkom *et al.* in the tegumental fraction of *S. mansoni* (van Balkom *et al.*, supplemental table 2a [3])

[e] Proteins identified by van Balkom *et al.* in both the tegumental and non-tegumental fraction of *S. mansoni* (van Balkom *et al.*, supplemental table 2c [3])

Supplementary Table 1C: Non-tegumental protein identifications present exclusively in the tPA-PPACK-biotin pull-down fraction and absent in the control fraction.

		Score		PSM	
Accession number	**Product**	**Control lane**	**Positive lane**	**Control lane**	**Positive lane**
Smp_138930.1	Glutamyl tRNA synthetase cytoplasmic	-	607	-	25
Smp_099310.1	Protein transport protein SEC23	-	452	-	9
Smp_033040.1	L lactate dehydrogenase	-	348	-	13
Smp_000030.2	26s proteasome regulatory particle subunit	-	303	-	12
Smp_034670.1	Tubulin gamma 1 chain	-	286	-	14
Smp_004910.1	RAB14, member RAS oncogene family	-	255	-	9
Smp_142630.1	Talin	-	243	-	4
Smp_123440.1	Cytoplasm protein	-	236	-	11
Smp_099800.1	Uncharacterized protein	-	204	-	11
Smp_101290.1	Actin protein 2, arp2	-	183	-	7
Smp_134860.1	Translational activator gcn1	-	181	-	8
Smp_213550.1	Pad1 homolog	-	171	-	6
Smp_095910.1	Signal recognition particle receptor subunit	-	162	-	8
Smp_003660.1	Spliceosome RNA helicase DDX39B	-	149	-	7
Smp_033250.1	Hypothetical protein	-	134	-	7
Smp_056890.1	Nitrilase	-	131	-	5
Smp_032260.1	Ribosomal protein L15	-	129	-	7
Smp_133390.1	CAP Gly domain containing linker protein 4	-	126	-	4
Smp_157410.1	Dynein heavy chain	-	123	-	8
Smp_194910.1	Saposin B domain containing protein	-	121	-	5
Smp_149040.1	Phosphorylase b kinase regulatory	-	119	-	5
Smp_210430.1	Ras protein Rab 1A	-	118	-	5
Smp_074020.1	AP 2 complex subunit alpha 2	-	106	-	4
Smp_145700.1	Ankyrin :unc	-	104	-	6
Smp_132690.1	RuvB protein 2	-	96	-	6
Smp_155720.1	Glycogen synthase kinase 3	-	92	-	5
Smp_015090.1	Coatomer subunit beta'	-	89	-	6
Smp_000870.1	FYVE, RhoGEF and PH domain containing protein 4	-	89	-	5
Smp_053220.2	Aldo keto reductase family 1, member B4	-	87	-	5
Smp_064050.1	Ruvb reptin and pontin	-	83	-	6
Smp_009580.1	Ubiquitin	-	75	-	5
Smp_079000.1	Charged multivesicular body protein 1b 2	-	70	-	4
Smp_149610.1	Hypothetical protein	-	63	-	5
Smp_099030.1	Casein kinase ii subunit alpha	-	53	-	4
Smp_049550.1	78 kDa glucose regulated protein	-	45	-	4

Supplementary Table 1D: Non-tegumental protein identifications present in both pull-down fractions, but enriched in the tPA-PPACK-biotin fraction compared to the control fraction.

		Score			PSM		
Accession number	**Product**	**Control lane**	**Positive lane**	**Ratio**	**Control lane**	**Positive lane**	**Ratio**
Smp_030730.1	Tubulin beta 2B chain	7065	16234	2.3	259	537	2.1
Smp_090120.2	Alpha tubulin	1633	4203	2.6	87	218	2.5
Smp_072330.1	Heat shock protein	233	2865	12.3	14	117	8.4
Smp_102040.3	Receptor for activated PKC	528	1530	2.9	20	64	3.2
Smp_147050.1	V type proton atpase catalytic subunit a	150	1196	8.0	4	37	9.3
Smp_018240.2	Transitional endoplasmic reticulum atpase	98	874	8.9	4	50	12.5
Smp_069130.1	Heat shock protein 105 kDa	49	695	14.1	2	18	9.0
Smp_119310.1	26S proteasome subunit RPT6	73	610	8.3	3	32	10.7
Smp_072870.1	Alpha centractin	58	579	9.9	7	26	3.7
Smp_090080.1	Estrogen regulated protein EP45	83	472	5.7	6	16	2.7
Smp_048560.1	Four and a half LIM domains protein 2	131	465	3.6	5	15	3.0
Smp_013470.2	small subunit ribosomal protein S2e	34	464	13.8	1	24	24.0
Smp_097660.1	Eukaryotic initiation factor 4A	82	456	5.6	5	29	5.8
Smp_042270.1	26S proteasome subunit ATPase 3	291	382	1.3	7	19	2.7
Smp_057320.1	Vesicle fusing ATPase 1	38	303	8.0	1	18	18.0
Smp_056360.4	P30 dbc protein	29	303	10.4	1	12	12.0
Smp_207010.1	Signal recognition particle 72 kDa subunit	44	247	5.6	1	10	10.0
Smp_071140.1	Adducin related protein	46	219	4.8	2	11	5.5
Smp_025510.1	Vacuolar protein sorting associated protein 4A	38	193	5.1	1	10	10.0
Smp_012470.1	26S proteasome subunit ATPase 2	42	174	4.1	2	9	4.5
Smp_059290.2	Dual specificity protein phosphatase 23	77	168	2.2	3	9	3.0
Smp_017070.1	26S proteasome subunit RPT4	30	157	5.2	2	13	6.5
Smp_138920.2	COP9 signalosome complex subunit	59	156	2.7	2	4	2.0
Smp_173840.1	26S proteasome subunit ATPase 4	35	140	4.0	1	4	4.0
Smp_011570.2	Ribosomal protein S4	24	120	5.1	2	10	5.0
Smp_170110.1	High density lipoprotein binding protein	46	100	2.2	3	6	2.0
Smp_001040.1	Kinesin heavy chain	21	98	4.8	1	8	8.0
Smp_058650.1	26S proteasome non ATPase regulatory subunit	43	95	2.2	2	6	3.0
Smp_072340.1	26S proteasome subunit RPT3	35	92	2.6	1	6	6.0
Smp_061650.1	26S proteasome non ATPase regulatory subunit	0	42	-	1	6	6.0

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7

CHAPTER 7

Fibrinogen and fibrin are novel substrates for *Fasciola hepatica* cathepsin L peptidases

Mirjam M. Mebius[1,2], Jody M. J. Op Heij[2], Aloysius G. M. Tielens[1,3], Philip G. de Groot[2], Rolf T. Urbanus[2], Jaap J. van Hellemond[1]

[1] Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam, the Netherlands
[2] Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[3] Department of Biochemistry and Cell Biology, Faculty Veterinary Medicine, Utrecht University, the Netherlands

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Abstract

Cathepsin peptidases form a major component of the secreted proteins of the blood-feeding trematodes *Fasciola hepatica* and *Schistosoma mansoni*. These peptidases fulfill many functions, from facilitating infection to feeding and immune evasion. In this study, we examined the *Fasciola* cathepsin L peptidases FhCL1, FhCL2, and FhCL3 and the schistosomal cathepsin peptidases SmCB1 and SmCL3 for their anticoagulant properties. Although no direct anticoagulant effect of these peptidases was observed, we discovered that cathepsin peptidases from *Fasciola*, but not from *Schistosoma*, were able to degrade purified fibrinogen, with FhCL1 having the highest fibrinogenolytic activity. Additionally, FhCL1 and FhCL2 both efficiently degraded fibrin. The lack of a direct anticoagulant or fibrinolytic effect of these peptidases is explained by their inhibition by plasma components. However, within the parasite gut, high concentrations of these peptidases could induce an anticoagulant environment, facilitating blood-feeding for extended periods.

Secreted peptidases play a crucial role in the life cycle of trematodes, both in the immature and mature stages. Of special interest are cathepsin peptidases, which are papain-like cysteine peptidases, that make up a large proportion of the transcriptome of trematodes (10-27%) [1] and their secretome [2]. These secreted cathepsin peptidases fulfill a broad range of functions from facilitating infection, acquisition of nutrients, tissue migration, and immune suppression [1]. In addition, secretomes of blood-feeding parasites, such as leeches or hookworm species, often contain a repertoire of peptides and peptidases with anticoagulant properties, to allow blood-feeding for extensive periods [3,4].

Fasciola and *Schistosoma* species are well studied examples of trematodes that feed on blood components. Cathepsins are the major components of the secretome of both *Fasciola hepatica* and *Schistosoma mansoni* [5–7]. In this study, we examined the anticoagulant capacities of several cathepsin peptidases from these blood-feeding trematodes: *F. hepatica* Cathepsin L 1 (FhCL1), FhCL2, FhCL3, *S. mansoni* Cathepsin B 1 (SmCB1), and *S. mansoni* Cathepsin L 3 (SmCL3). Cathepsin L peptidases are the most abundantly secreted peptidases from *F. hepatica*. FhCL3 is known to cleave collagen and is mostly secreted by newly excysted juveniles (NEJs) upon penetration through the gut wall, accounting for 37% of the NEJs secretome [7]. FhCL1 and FhCL2 are major components of bile duct-residing, adult parasite secretions, and represent 69% and 22% of the adult secretome, respectively [7]. They are known to be involved in the cleavage of hemoglobin, collagen, IgG, fibronectin, and laminin [8–11]. SmCB1 is the most abundant cysteine peptidase in adult *S. mansoni* residing in the mesenteric veins and is, like FhCL1 and FhCL2, involved in the cleavage of hemoglobin, IgG, serum albumin, but also α-2-macroglobulin [5,12]. SmCL3 is a gut-associated, but not secreted, peptidase of schistosomula and adult *S. mansoni* and is involved in the digestion of host proteins such as serum albumin and hemoglobin [13]. All five peptidases were recombinantly expressed in *Pichia pastoris* and activated as previously described [6,13–15]. Anticoagulant properties of these peptidases were first examined with clot lysis time assays [16], with thrombin generation assays [17], and with fibrin formation assays in which fibrin was formed in 3x diluted trisodiumcitrate anticoagulated plasma by addition of 4 μM phospholipids (40% phosphatidylcholines, 40% phosphatidylethanolamines, and 20% phosphatidylserines), 10 mM $CaCl_2$, in the absence or presence of tissue factor (1 pM or 5 pM). However, no anticoagulant effect of the peptidases was found with these assays (data not shown).

In order to assess whether the expressed peptidases may have a fibrinolytic effect, cleavage of the plasmin substrate Boc-val-leu-lys-AMC was examined (Fig. 1A). FhCL1, FhCL2 and SmCB1 were able to cleave the plasmin substrate, generating a fluorescent signal. This indicates a potential fibrinolytic activity of these peptidases.

To examine this potential fibrinolytic activity, lysis of fibrin was assessed for the *F. hepatica* and schistosomal peptidases on a fibrin gel using plasmin as a positive

control (Fig. 1B, C). FhCL1 and FhCL2 demonstrated lysis of the fibrin gel after the incubations as visible by the formation of transparent circles (Fig. 1B). FhCL3 did not lyse the fibrin gel, consistent with the absence of cleavage of the plasmin substrate (Fig. 1B). We also found that none of the schistosomal peptidases was able to lyse the fibrin gel within 24 hours (Fig. 1C), despite the ability of SmCB1 to cleave plasmin substrate (Fig. 1A).

Next, fibrinogenolytic activity of the studied cysteine peptidases was assessed through analysis of the hydrolysis of purified fibrinogen with SDS-PAGE, as described previously [18] (Fig. 1D-H), and compared to cleavage of fibrinogen by plasmin (Fig. 1i). Interestingly, all three tested *F. hepatica* peptidases were able to cleave fibrinogen, as demonstrated by degradation of the fibrinogen α-chain (66 kDa), β-chain (52 kDa) and γ-chain (46.5 kDa) (Fig. 1D-F). FhCL1 has a larger fibrinogenolytic activity than FhCL2 and FhCL3 and is capable of degradation of the fibrinogen α-chain, β-chain, and γ-chain. FhCL2 and FhCL3 demonstrate only minor cleavage of the γ-chain and slower cleavage of the α-chain and β-chain compared to FhCL1. For all *F. hepatica* peptidases cleavage of the α-chain proceeded faster than cleavage of the β-chain and γ-chain. The cleavage pattern of the *F. hepatica* peptidases showed major differences with that of plasmin, indicating distinct cleavage sites of these peptidases. Plasmin seemed to degrade first the β-chain and γ-chain, with the α-chain still present after 4h of incubation. Lastly, consistent with the lack of fibrinolytic activity, both schistosomal peptidases did not demonstrate any fibrinogenolytic activity (Fig. 1G,H).

These results show that FhCL1 compared to FhCL2, is much more active in degrading the plasmin-substrate (Fig. 1A) and fibrinogen (Fig. 1D,E), but their activity towards fibrin is similar (Fig 1B). Therefore, the activity of these peptidases is strongly dependent on the actual substrate. Differences in substrate specificity have been described for the *F. hepatica* peptidases [6], where FhCL1 most efficiently cleaves substrates with hydrophobic residues at the P2 position (including Z-FR-NHMec and Boc-VLK-NHMec) while FhCL2 and FhCL3 have a preference for Pro-containing substrates and they cleave substrates with Phe and Leu in the P2 position much less effectively. Likewise, different K_m values of SmCB1 and SmCL3 for the Z-FR-AMC substrate have been described [12,13], indicating differences in substrate specificity for substrates with a hydrophobic residue at the P2 position.

From these experiments it is clear that the studied *F. hepatica* peptidases, but not the schistosomal peptidases, contain both fibrinogenolytic (FhCL1, FhCL2 and FhCL3) and fibrinolytic (FhCL1 and FhCL2) activity on purified fibrinogen and fibrin. This, however, contrasts with our first findings that these peptidases do not affect fibrin formation and clot lysis times in plasma. Therefore, we next sought an explanation for this discrepancy by examining the effect of plasma on the proteolytic activity of these peptidases (Fig. 2). Proteolytic activity of FhCL1, FhCL2, FhCL3, and SmCL3 on fluorogenic

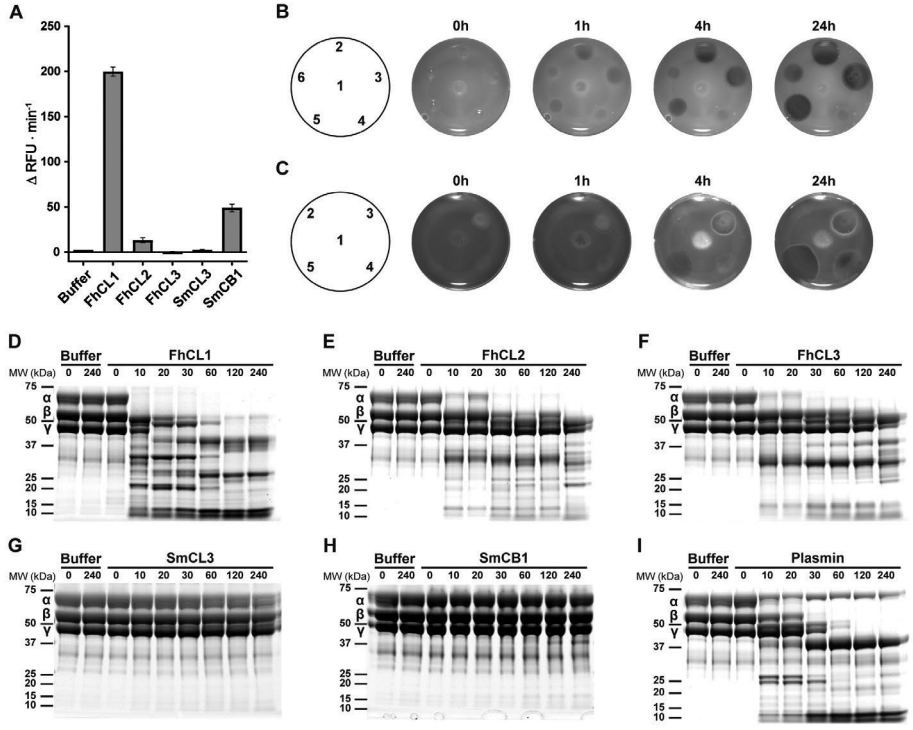

Figure 1. Fibrinogenolytic and fibrinolytic activities of secreted peptidases of *F. hepatica* and *S. mansoni*.

A) Cleavage of plasmin substrate (Boc-Val-Leu-Lys-AMC, Bachem, Bubendorf, Switzerland) in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) by 20 nM of peptidases of *F. hepatica* (FhCL1, FhCL2, and FhCL3) and *S. mansoni* (SmCL3 and SmCB1). Recombinant peptidases were produced in *Pichia pastoris* and purified as previously described [6,13,14] and activated for 10 min at 37°C in reaction buffer (2.5 mM EDTA, 0.1 M Sodium Citrate, 2 mM DTT, pH 5.0). SmCB1 was activated with activation buffer (0.1M sodium acetate, 0.1% PEG6000, 1 mM EDTA, 2.5 mM DTT, 10 mg/ml Dextran sulphate (Mw 500 kDa), pH 4.5) for 2 h at 37°C [15]. Fluorescence was monitored for 120 minutes at 37°C on a Spectromax M^2 fluorescence microplate reader (MTX Lab Systems, Bradenton, FL, USA) equipped with a 390 nm excitation filter and 460 nm emission filter. Initial rates of fluorescence are represented as mean ± SD. All values represent three replicates of duplo measurements. Fibrinolytic activity of *F. hepatica* (B) and schistosomal (C) peptidases was assessed on a fibrin gel (representative image of three experiments). Fibrin gels were prepared by coagulating 4.9 mg/ml purified human fibrinogen (Enzyme research laboratories, South Bend, IN, USA) in HBS with 1 U/ml human alpha-thrombin (Haematologic Technologies, Essex Junction, VT, USA) followed by addition of peptidase samples and subsequent incubation at 37°C for up to 24 h. B) Fibrinolytic activity of 10 µl aliquots of reaction buffer (2.5 mM EDTA, 0.1 M Sodium Citrate, 2 mM DTT, pH 5.0) (1), FhCL1 (10 µM in reaction buffer) (2), FhCL2 (10 µM in reaction buffer) (3), FhCL3 (10 µM in reaction buffer) (4), plasmin (streptokinase-activated plasminogen, 100 µg/ml in reaction buffer) (5), and plasmin (streptokinase-activated plasminogen, 10 µg/ml in reaction buffer) (6), spotted on a fibrin gel. C) Fibrinolytic activity of 10 µl aliquots of reaction buffer (1), SmCL3 (3 µM in reaction buffer) (2), SmCB1 (250 nM in reaction buffer) (3), plasmin (streptokinase-activated plasminogen, 100 µg/ml in reaction buffer) (4), and plasmin (streptokinase-activated plasminogen, 10 µg/ml in reaction buffer) (5) spotted on a fibrin gel. D-I) Fibrinogenolytic activity of *F. hepatica* and schistosomal secreted peptidases and plasmin was determined on Coomassie brilliant blue stained 10% SDS-PAGE gels with a modified

fibrinogenolytic assay [18]. Purified fibrinogen (2 µg/ml in HBS) was incubated at 37°C with 100 nM FhCL1 (D), 100 nM FhCL2 (E), 100 nM FhCL3 (F), 100 nM SmCL3 (G), 25 nM SmCB1 (H), or 100 nM Plasmin (streptokinase-activated plasminogen) (I), respectively. Reactions were terminated by adding denaturing solution (SDS-Page sample buffer containing 6.7 M urea, 2% SDS, and 25 mM dithiothreitol) and heating at 100 °C for 10 min (representative image of three experiments). The numbers at the top of each lane represent the time (in minutes) when samples were taken during the digestion.

peptide substrates was examined in the presence of either citrated or heparinized plasma (Fig. 2A-D). Since SmCB1 was activated by dextran sulphate [15] and dextran sulphate is a non-physiological trigger for contact system activation *in vitro*, factor XII depleted citrated plasma was used to examine proteolytic activity of SmCB1 in plasma (Fig. 2E). Examination of proteolytic activity showed that all five examined peptidases were inhibited by plasma components at low concentrations (< 0.1% plasma). This inhibition of the peptidases by plasma components explains the absence of anticoagulant effects observed in the performed clot lysis and thrombin generation assays.

Reduced glutathione (GSH) has been described as an activating agent for FhCL1 through reduction of the active site cysteine [19]. Therefore, we also examined whether addition of GSH to plasma could counteract the inhibitory effect of plasma components for FhCL1 and FhCL2 (Fig. 2F,G). However, addition of GSH did not reverse the effect of plasma components. Most likely the GSH did not have an additional effect to the DTT that was already present in the buffer used to activate the peptidases.

In the present study, we report the discovery of fibrinogen and fibrin as novel substrates for the *F. hepatica* cathepsin peptidases FhCL1 and FhCL2 and the fibrinogenolytic activity of FhCL3. We also examined two schistosomal cathepsin peptidases, SmCL3 and SmCB1, for their fibrinogenolytic and fibrinolytic activity and found that fibrinogen and fibrin are not substrates for these peptidases. As all tested peptidases are inhibited by plasma components, we suggest that they most likely do not affect blood coagulation in their host. However, secretion of *F. hepatica* cathepsin L peptidases at high concentrations in the parasite gut implies that this may locally overcome the inhibitory effect of plasma components and induce an anticoagulant environment in the gut through cleavage of fibrinogen and fibrin, allowing blood-feeding by the parasite for extended periods. Our finding that schistosomal peptidases are not able to cleave fibrinogen or fibrin is in line with the presence of other anticoagulant and fibrinolytic strategies adopted by schistosomes (reviewed in [20]). This allows schistosomes to counteract fibrin formation at their surface, allowing blood-feeding and survival of the parasite within its host.

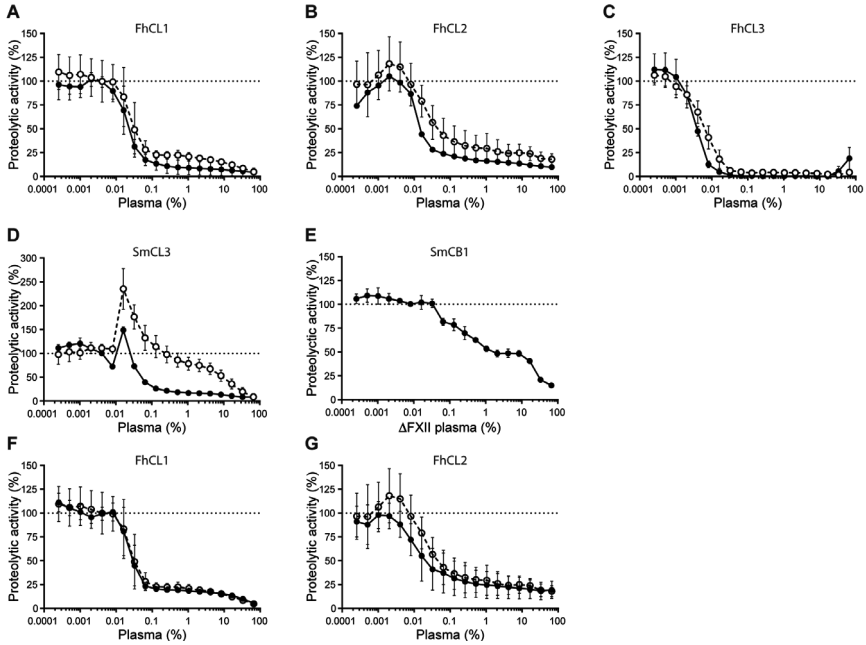

Figure 2. Proteolytic activity of *F. hepatica* and schistosomal secreted peptidases is inhibited by human plasma.

Proteolytic activity of FhCL1 (20 nM) (A) or FhCL2 (20 nM) (B) on Boc-Val-Leu-Lys-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of citrated (open circles) or heparinized human platelet poor plasma from healthy donors (closed circles). C) Proteolytic activity of FhCL3 (20 nM) on Z-Pro-Arg-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of citrated (open circles) or heparinized human platelet poor plasma from healthy donors (closed circles). D) Proteolytic activity of SmCL3 (20 nM) on Z-Phe-Arg-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of citrated (open circles) or heparinized human platelet poor plasma from healthy donors (closed circles). Proteolytic activity of dextran sulfate-activated SmCB1 (20 nM) on Z-Phe-Arg-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of FXII depleted human plasma. Proteolytic activity of FhCL1 (20 nM) (F) or FhCL2 (20 nM) (G) on Boc-Val-Leu-Lys-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of citrated human platelet poor plasma from healthy donors without (open circles) or with addition of glutathione (3.3 mM of which 90% was the reduced form) (closed circles). Fluorescence was monitored for 120 minutes at 37°C on a Spectromax M^2 fluorescence microplate reader (MTX Lab Systems, Bradenton, FL, USA) equipped with a 390 nm excitation filter and 460 nm emission filter. Proteolytic activity is displayed as a percentage (mean ± SD) of the activity without addition of plasma (100%, dotted line). All values represent three replicates of duplo measurements.

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8

CHAPTER 8

Summarizing discussion

Parasitic worms interact with their host on multiple levels and often for prolonged periods. The parasite interacts with a variety of host cells during migration, acquisition of nutrients, and reproduction. Additionally, parasites are continuously exposed to the host immune system and have to evade ongoing immune responses. Furthermore, blood-dwelling and blood-feeding parasites interact for prolonged periods with the haemostatic system of their host. Indeed, changes in blood coagulation have been observed in infections with various helminths, including *Schistosoma* and *Fasciola* [1,2]. These helminths, as well as other blood-feeding or blood-dwelling parasites, have been proposed to possess a variety of molecular mechanisms to modify their host's coagulation system. The work described in this thesis provides (1) an overview of the currently identified blood coagulation modification mechanisms of schistosomes, (2) insight in coagulopathy in urinary schistosomiasis without hepatic dysfunction, and (3) identification of novel mechanisms of coagulation modification by the parasitic helminths *S. mansoni* and *F. hepatica*. These findings will contribute to a better understanding of the complex interactions between host haemostasis and parasitic helminths.

Coagulation modification by schistosomes: current status

Chapter 2 provides an overview of currently known mechanisms of coagulation modification by schistosomes. The large variety of mechanisms that are equipped by the parasite, demonstrates that the parasite has a remarkably strong ability to modify the host haemostatic response at all levels. In order to modify host haemostasis, the parasite uses schistosome-specific mechanisms that inhibit key steps in coagulation and exploits or mimics host mechanisms for regulation of haemostasis.

This overview of coagulation modification raised the question how complete our current knowledge of coagulation modification by schistosomes is, and whether it is likely that schistosomes possess yet unknown coagulation modification strategies. In the five years after the review in **Chapter 2** has been published, the research on the interactions between schistosomes and haemostasis has continued, which has led to novel insights and discoveries in this field. It therefore goes without saying that the overview presented in this thesis is now incomplete and requires some updating.

Several novel molecular mechanisms have been described in adult schistosomes that are proven or suggested to affect haemostasis. It has been described that *S. mansoni* can cleave both fibronectin and high-molecular weight kininogen (HK). Fibronectin is a plasma protein that is incorporated into blood clots and increases stability of platelet aggregates [3]. *S. mansoni* has been demonstrated to cleave both purified fibronectin and plasma fibronectin through a surface associated cysteine peptidase

activity [4]. Two calpains, named SmCalp1 and SmCalp2, were recently discovered on the outer surface of the adult *S. mansoni* tegument [4]. These calpains are suggested to be involved in both the cleavage of fibronectin [4] and HK [5]. HK is a co-factor of the contact activation pathway and acts as a scaffold to localize coagulation factor XII (FXII), FXI, and plasma kallikrein (PK) in close proximity of each other on anionic surfaces. Cleavage of HK by PK at two cleavage sites results in the release of the peptide bradykinin, that stimulates vasodilatation and the release of tissue-type plasminogen activator (tPA) from the endothelium [6,7]. Additionally, the two cleavages of HK by PK convert HK into a two-chain form, which has increased affinity for anionic surfaces [8]. The cleavage of HK by schistosomal cysteine peptidases is different from cleavage by PK, resulting in different degradation fragments and no bradykinin generation [5]. A secreted trypsin-like serine peptidase from adult *S. mansoni*, SmSP2, has also been demonstrated to cleave HK, resulting, in contrast to the cysteine peptidase activity, in the release of the vasodilatory peptide bradykinin [9]. It was also shown that SmSP2 inactivates the vasoconstrictor vasopressin and degrades fibronectin [9]. Furthermore, SmSP2 cleaves tPA into a more active double-chain tPA form and activates plasminogen to plasmin, thereby both indirectly and directly enhancing fibrinolysis [9]. Orthologues of SmSP2 can be found in *S. japonicum* and *S. haematobium* [9]. Enhancement of fibrinolysis has also been shown for another protein of *S. mansoni*. The tegument associated *S. mansoni* enolase (SmEno) has been demonstrated to bind plasminogen and enhance its activation to plasmin in the presence of tPA [10]. However, knock-down of expression of SmEno did not affect the capacity of the parasite to enhance fibrinolysis, suggesting redundancy in schistosomal mechanisms that enhance fibrinolysis [10].

In addition to several novel peptidases of schistosomes that interact with haemostasis, several peptidase inhibitors of schistosomes have been described to inhibit components of the haemostatic system and/or affect coagulation times. A Kunitz-type peptidase inhibitor, SmKI-1, localized in the tegument and present in excretory/secretory products of *S. mansoni*, was found to inhibit FXa and PK, but not thrombin [11]. It delayed blood clot formation approximately three-fold, as reflected by increased activated partial thromboplastin time (APTT; contact activation/intrinsic pathway) and prothrombin time (PT; extrinsic pathway) [11]. A Kunitz-type peptidase inhibitor, SjKI-1, was also identified in *S. japonicum* [12]. SjKI-1 is highly transcribed in adults and eggs, but could only be detected by immunolocalization in eggs trapped in the infected mouse intestine wall. [12]. Like SmKI-1, SjKI-1 inhibits FXa and PK, but not thrombin. [12]. SjKI-1 prolonged APTT by approximately two-fold, but did not affect coagulation times of the extrinsic pathway [12]. Both Kunitz-type peptidase inhibitors, SmKI-1 and SjKI-1, also inhibited chymotrypsin, trypsin, and neutrophil elastase [11,12]. A second peptidase inhibitor from *S. japonicum*, SjSPI, is highly expressed in the egg-

laying adult worm [13]. SjSPI is a serine peptidase inhibitor and inhibits thrombin, chymotrypsin, and trypsin [13]. However, the biological significance of SjSPI is not clear, since it is not secreted and it is continuously expressed from schistosomula to adult worms. As its expression is highest in the egg and adult stages, a possible role in maintaining egg physiology or regulation of endogenous serine peptidases has been suggested and effects on blood coagulation have not been studied [13].

Besides interactions between adult schistosomes and haemostasis, interactions between other schistosome life stages and haemostasis have also been studied. A study on the interaction between the eggshell and plasma components has led to the finding that the eggshell binds VWF, fibrinogen, and fibronectin [14]. This interaction is suggested to facilitate adherence of the eggs to the endothelium and mediate subsequent extravasation of eggs [14]. Also, binding of plasminogen and enhancement to plasmin does not seem to be restricted to the adult parasite. Secretions by *S. mansoni* cercariae contain the protein SmVAL18, that has been demonstrated to bind plasminogen and enhance its conversion to plasmin in the presence of urokinase-type plasminogen activator [15]. In contrast to the adult parasite where enhancement of plasmin activation is linked to fibrinolysis of blood clots, it is suggested that the activation of plasmin by cercariae aids in degradation of extracellular matrix components in order to facilitate invasion of the human host.

Apart from discovery of novel mechanisms for coagulation modification, several studies have also provided further insight into already known mechanisms for coagulation modification. It is well-known that schistosomes express a number of enzymes that catabolize extracellular ATP to adenosine, which has been suggested to interfere with platelet activation during schistosomiasis, as described in **Chapter 2**. Adenosine is an inhibitor of ADP-induced platelet aggregation and has immunoregulatory effects, which are mostly anti-inflammatory. The schistosomal enzymes that convert ATP to adenosine include alkaline phosphatase (SmAP), phosphodiesterase (SmNPP-5), and ATP-diphosphohydrolase (SmATPDase1). Based on homology with human enzymes, it was implied that all of these enzymes participate in the degradation of ATP and ADP, and only SmAP and SmNPP-5 would degrade AMP. Research into the relative importance of these three different enzymes in ATP, ADP, and AMP conversion, was performed by knocking-down these enzymes [16]. Only the knock-down of SmATPDase1 significantly impaired the ability of the worm to catabolize ATP or ADP, implying that the cleavage steps of ATP and ADP are, unlike these steps in humans, non-redundant in schistosomes [16]. In another study, knock-down of SmAP has been described to greatly reduce the capacity of the parasite to degrade AMP, suggesting that SmAP , and not SmNPP-5, is the major enzyme for the conversion of AMP to adenosine [17]. It was therefore concluded that SmNPP-5 does not participate in the cleavage of ATP, ADP, or AMP, and that the function of this enzyme at the worm

surface is unknown [16]. However, it has been described that parasites in which the SmNPP-5 gene is suppressed, fail to establish a robust infection in mice, indicating that SmNPP-5 does fulfil an important role during infection [18]. A more recent study challenged this conclusion, by demonstrating that SmNPP-5 is capable of hydrolysing ADP to inorganic phosphate and that recombinant SmNPP-5 strongly inhibits both ADP and collagen induced platelet aggregation [19]. The lack of effect of knock-down of SmNPP-5 on the capability to cleave ADP is attributed to the fact that SmATPDase1 is found in approximately 5-fold greater relative abundance than SmNPP-5 and this does not exclude a function of SmNPP-5 in regulation of extracellular ADP [19].
Further studies on schistosomes and haemostasis will, without doubt, lead to further understanding of coagulation modification mechanisms of schistosomes and it is to be expected that novel coagulation modification strategies will be discovered. This is already demonstrated by the content of this thesis, as **Chapter 4** of this thesis describes a novel interaction between adult schistosomes and VWF, and **Chapter 6** provides further insight into the capacity of schistosomes to enhance fibrinolysis.

Coagulopathy in schistosomiasis: lessons from urogenital schistosomiasis

Having gained more insight into coagulation modification mechanisms of schistosomes in **Chapter 2**, we next focussed on the consequences of coagulation modification and schistosomiasis on haemostasis in schistosomiasis patients. Clinical studies have demonstrated that patients with hepatosplenic schistosomiasis develop complex haemostatic abnormalities, linked to the risk of fatal bleeding from gastro-oesophageal varices [1]. These haemostatic abnormalities are characterized by decreased levels of both procoagulant and anticoagulant factors, impaired coagulation, ongoing thrombin generation, and continuous fibrinolysis, as described in more detail in **Chapter 3** and reviewed in detail by Tanabe [1]. The liver plays a key role in haemostasis as it is the site of both synthesis and clearance of most of the proteins involved in coagulation and fibrinolysis, as well as their inhibitors [20]. Advanced hepatic disease will therefore result in alterations in haemostasis [20]. Nearly all of the studies investigating coagulation changes in schistosomiasis have been performed in patients suffering from chronic schistosomiasis with hepatic or hepatosplenic pathology. Insight into the contribution of the parasitic infection to the observed haemostatic abnormalities is therefore lacking. In **Chapter 3** we have addressed this by studying haemostatic changes in a small number of schistosomiasis haematobium patients without advanced hepatic disease. In the absence of hepatic disease, inflammation-mediated endothelial activation results in increased secretion

of VWF by the endothelium. Despite the fact that plasma levels of VWF are increased, coagulation and fibrinolysis are not activated in these patients, reflected by unaffected levels of thrombin-antithrombin complex and D-dimers. Although the small size of the study described in **Chapter 3** forms a limitation in the generalization of the results to all schistosomiasis patients, it still provides the first evidence that the parasite itself does not cause the major haemostatic changes observed in hepatosplenic schistosomiasis. However, endothelial activation as a result of the parasitic infection could be involved in initiation of coagulation abnormalities in hepatosplenic schistosomiasis. Larger studies into coagulation abnormalities in schistosomiasis without advanced hepatic disease are needed to confirm this.

This raises the question what the contribution of hepatic pathology, the parasitic infection, and coagulation modification by the parasite is to the observed coagulation changes in hepatosplenic schistosomiasis. Patients with liver disease frequently develop complex haemostatic abnormalities, which can lead to both thrombosis and bleeding complications. These haemostatic abnormalities can be described as "rebalanced haemostasis". Decreased hepatic function results in parallel decreased production of both prothrombotic and antithrombotic coagulation factors [20,21]. Thrombin generation is thus preserved in liver disease, and in advanced liver disease even hypercoagulation is observed [21]. In schistosomiasis, there is also evidence of elevated thrombin generation, as demonstrated by increased levels of thrombin-antithrombin complex [1]. In liver disease, decreased platelet counts are compensated by increased VWF levels [20], which is also observed in schistosomiasis [22] and described in **Chapter 3**. This rebalanced haemostatic system is however easily knocked out of balance when the system is stressed, for example during infection, resulting in bleeding or thrombotic complications [20,23]. Although bleeding from gastro-oesophageal varices also occurs in liver disorders, it is believed that this is not a result of altered haemostasis, but rather the result of portal hypertension combined with local vascular abnormalities, as both anticoagulant and procoagulant therapy do not affect the severity of bleeding [21,24–26]. Also, a hyperfibrinolytic state is described in patients with liver disease [20,21] and also in schistosomiasis [1], although it remains uncertain whether this is true accelerated fibrinolysis, or whether it reflects decreased hepatic clearance of markers of fibrinolysis, such as D-dimer [1,21].

Thus, in liver disease there is a state of rebalanced haemostasis that is more easily tipped out of balance when the system is stressed, for example during infection [23]. The coagulopathy observed in schistosomiasis has all the characteristics of rebalanced haemostasis, where the parasitic infection and the resulting immune response could bring the system out of balance. The highly procoagulant nature of the eggshell [14,27] and pro-inflammatory cytokines produced during infection, such as TNF-α and IL-1β [28,29], could trigger inflammation-mediated endothelial activation, as

described in **Chapter 3**, leading to the release of VWF from the endothelium [30]. The local anticoagulant mechanisms of schistosomes are not expected to affect systemic haemostasis. Thromboelastography experiments on murine blood pre-incubated with *S. mansoni* and blood from mice infected with the parasite, provided some more insight into the effects of schistosomes on local and systemic coagulation [31]. These experiments revealed that blood from infected mice shows hypercoagulation, leading to faster clot formation, but with less stable clots formed, reflecting lower blood platelet numbers and lower levels of antifibrinolytic proteins leading to hyperfibrinolysis. This mirrors the haemostatic abnormalities observed in hepatosplenic schistosomiasis in humans [1]. Interestingly, these coagulation changes do not occur in early infection, but are only detected after considerable organ pathology as result of entrapment of eggs is present, strongly indicating that the haemostatic abnormalities result from liver disease [31]. This is consistent with the lack of increased coagulation activation and the lack of increased fibrinolysis activation that was observed in schistosomiasis haematobium patients without hepatic disease, as described in **Chapter 3**. However, pre-incubation of murine blood with schistosomes, resulted in remarkably different coagulation changes. Blood clots were formed slower and clot stability is not affected. This indicates that schistosomes act as strong local anti-coagulants, without significant effects on systemic haemostasis [31]. However, direct effects of schistosomes on fibrinolysis were not determined in this study, and it could be that schistosomes contribute to the hyperfibrinolysis observed in chronic schistosomiasis.

The occurrence of coagulopathy in hepatosplenic schistosomiasis raises the question whether we should treat haemostatic abnormalities with anticoagulants or antifibrinolytic drugs. As the parasitic infection forms the trigger that brings the rebalanced haemostatic system out of balance, it is expected that treatment of the infection with praziquantel resolves haemostatic abnormalities. Indeed, for bacterial infections in cirrhosis patients it was shown that successful treatment of the infection restored haemostatic parameters to pre-infection levels in 5 days [32]. Furthermore, liver fibrosis, but not cirrhosis, is often a reversible process, so hepatic function can be restored in fibrosis patients. It has been demonstrated that praziquantel itself may also have anti-fibrotic effects and could thus contribute even further to restore hepatic function, although effects on haemostatic parameters were not examined in this study [33]. Furthermore, it is interesting to consider whether the use of anticoagulation, procoagulant treatment, or antifibrinolytic treatment should be used to prevent thrombosis or bleeding complications in schistosomiasis. In liver fibrosis and cirrhosis, preventative anticoagulation has been studied in the prevention of portal vein thrombosis [21,34,35]. However, since portal vein thrombosis only occurs in approximately 5% of hepatosplenic schistosomiasis patients [36], the application of preventative anticoagulation in all hepatosplenic schistosomiasis patients without

further identification of the patients at risk for portal vein thrombosis would most likely not be beneficial. Administration of procoagulant drugs in order to prevent bleeding from gastro-oesophageal varices is unlikely to be beneficial to schistosomiasis patients, as a study in cirrhosis patients demonstrated that administration of FVIIa did not affect severity and outcome of variceal bleeding [21,26], and the occurrence of variceal bleeding is most likely linked to portal hypertension and local vascular abnormalities instead of hypocoagulation [21]. Administration of antifibrinolytic drugs has been useful in some cirrhosis cases in the management of active bleeding [21]. However, it has not been well studied whether prophylactic treatment with antifibrinolytic drugs could prevent bleeding in patients with liver disease [21]. Although a hyperfibrinolytic state is present in hepatosplenic schistosomiasis patients, it therefore remains unknown whether antifibrinolytic treatment could be applied to prevent bleeding in patients. In conclusion, early treatment with praziquantel in order to clear the infection, thereby taking away the trigger for the haemostatic imbalance, would be preferred over preventative treatment that affects the haemostatic system.

Modification of blood coagulation: novel mechanisms

The research presented in this thesis has led to the discovery of several novel mechanisms used by helminth parasites for modification of blood coagulation, presented in **Chapters 4 and 6**. Additionally, the study described in **Chapter 5** provides more insight into regulation of the VWF-cleaving peptidase ADAMTS13 during normal haemostasis.

Targeting VWF: alternative routes to control multimer size

S. mansoni *VWF-cleaving peptidase*

Schistosome eggs are known to interact with plasma VWF [14], however, the interaction between VWF and the adult life stage of schistosomes has not been investigated. Plasma levels of VWF are elevated in schistosomiasis, without apparent thrombosis, as described in **Chapter 3**. VWF binds platelets at sites of endothelial damage and is thereby involved in initiation of the formation of a platelet plug [37]. The apparent lack of coagulation activation, despite elevated VWF levels, suggest that schistosomes may counteract increased VWF secretion. In **Chapter 4** we have investigated the interactions between *S. mansoni* and VWF and discovered that SmCB2, a known cysteine peptidase of *S. mansoni*, can cleave VWF. The finding of an *S. mansoni* VWF-cleaving peptidase suggests that while the eggs require binding of VWF for extravasation, the adult worm actively degrades VWF to create a local anticoagulant environment around itself, which may explain the lack of platelet binding to the outer surface of the adult worm [27].

SmCB2 is a cysteine peptidase of *S. mansoni*, that is localized in the tegumental outer-surface structure of the adult worm [38]. The proteolytic activity of SmCB2 towards the FRETS-VWF73 substrate demonstrates that SmCB2 has the capacity to cleave VWF *in vitro*. Additional experiments need to be performed in order to examine this capacity under more physiological conditions. First the proteolytic activity of SmCB2 towards purified, native VWF will be examined. Additionally, the use of a flow chamber model of VWF-string formation on either HEK293 cells or blood outgrowth endothelial cells would allow visualization of degradation of VWF by SmCB2 under flow conditions and would be an interesting addition to the characterization of this peptidase [39,40].
Discovery of a novel VWF-cleaving peptidase could have implications beyond schistosomiasis, for example in disorders where regulation of VWF is disturbed. In **Chapter 4** we have shown that the peptidase is not sensitive to components specific for TTP patient plasma. In these patients regulation of VWF is disturbed as a result of a deficiency of the VWF-cleaving peptidase ADAMTS13. This ADAMTS13 deficiency can be congenital, caused by mutations in the ADAMTS13 gene, or more often idiopathic, caused by inhibition of ADAMTS13 activity or enhanced clearance through binding of autoantibodies directed at ADAMTS13 [41,42]. As we have shown that the *S. mansoni* VWF-cleaving peptidase is not sensitive to autoantibodies present in TTP patient plasma and is likely to have low immunogenicity as reflected by the longevity of the parasite, we postulate that SmCB2 can have applications in the treatment of TTP.
The applicability of SmCB2 in TTP could be assessed in an animal model for TTP. Several different mice models of TTP have been developed and a baboon model for acquired TTP is available [43]. ADAMTS13-deficient mice do not spontaneously develop TTP symptoms, but require an additional trigger such as Shiga toxin or recombinant UL-VWF, in order to develop TTP symptoms [43]. Acquired TTP can be induced in baboons and mice through administration of an inhibitory antibody against ADAMTS13 [43,44]. In baboons this results, without the need for an additional trigger, in the development of early-stage TTP symptoms, without progression to end-stage disease [43]. However, in mice an additional trigger is required for the development of symptoms [44]. The UL-VWF-induced congenital and acquired TTP models in mice and the baboon model for acquired TTP have been used to test novel therapies for TTP [44–46], and could similarly be of value to test the therapeutic value of SmCB2 in congenital and acquired TTP.

Plasmin as activator of ADAMTS13

Having discovered an alternative route employed by *S. mansoni* to control VWF multimer length, we next investigated alternative routes for control of VWF functionality during normal haemostasis in **Chapter 5**. The fibrinolytic protein plasmin has been demonstrated to cleave VWF and has been proposed as a backup for ADAMTS13 [47].

Additionally, amplified plasmin activity resolved TTP symptoms in a mouse model of acquired TTP [44]. In contrast to the positive effects of plasmin, several reports indicate that plasmin may cause proteolytic inactivation of ADAMTS13, which has been suggested to present a risk of TTP [48–50]. In **Chapter 5** we demonstrated that plasmin truncates ADAMTS13, which in contrast to earlier reports, increases the activity of ADAMTS13. Differences in methodology may account for the observed differences in the effect of plasmin on ADAMTS13. In the previously reported studies, ADAMTS13 was incubated for prolonged periods with plasmin, 2 – 24 hours, before assessment of functionality of the peptidase [48–50], while we incubated plasma only for 30 minutes in the presence of plasmin. The first stages of plasmin cleavage of ADAMTS13 take place at the C-terminus [49], which as we demonstrated in **Chapter 5**, results in increased ADAMTS13 activity. However, it is possible be that prolonged incubation (hours) of ADAMTS13 with plasmin results in additional cleavage of ADAMTS13 at different sites, eventually resulting in loss of activity [48,50].

The increased ADAMTS13 activity in plasmin cleaved ADAMTS13 is an interesting finding, because it provides new insights into regulation of ADAMTS13 activity. Until recently, it was believed that ADAMTS13 was present in plasma as an active enzyme, which activity was regulated solely by the conformational changes in its substrate, VWF. This view changed with identification of the existence of two conformations of ADAMTS13, closed and open, with different levels of proteolytic activity [51–53]. In its closed, less active conformation the C-terminal CUB domains of ADAMTS13 interact with its spacer region, causing ADAMTS13 to fold upon itself [51]. VWF binding through the CUB domains leads to conformational changes in ADAMTS13, a process called conformational activation, resulting in the open conformation of ADAMTS13 which is proposed to have increased proteolytic activity [51]. We propose that C-terminal truncation of ADAMTS13 by plasmin, removing the eighth thrombospondin type-1 repeat and both CUB domains, causes ADAMTS13 to adopt an open conformation, resembling the conformational activation of ADAMTS13 and resulting in increased proteolytic activity. This discovery implies that not only substrate binding is involved in regulation of ADAMTS13 activity, but that also the plasma enzyme plasmin can regulate the proteolytic activity of ADAMTS13. This could reflect a physiological mechanism to enhance ADAMTS13 activity at sites of microthrombus formation. This would suggest two additional roles for plasmin at sites of microthrombus formation, besides the degradation of fibrin clots: 1) enhancing ADAMTS13 activity, thereby indirectly enhancing VWF cleavage, and 2) direct cleavage of VWF by plasmin [47].

Further research into the proteolytic regulation of ADAMTS13 by plasmin is required to identify the exact cleavage sites of plasmin, thereby confirming that indeed the eighth thrombospondin type-1 repeat and both CUB domains are removed from ADAMTS13, which would convert ADAMTS13 to a more open conformation. Additionally, it would

be interesting to see if the increased activity of the truncated ADAMTS13 towards FRETS-VWF73 can be confirmed using a more physiological substrate, such as multimeric VWF, or in a flow chamber model of VWF-string formation [39,40].

Effects of helminths on fibrinogen, fibrin, and fibrinolysis

S. mansoni *tPA binding proteins*

The serine peptidase plasmin is the main enzyme of the fibrinolytic system, where it breaks down formed thrombi through degradation of fibrin [54]. However, plasmin is a versatile peptidase, as demonstrated in **Chapter 5**, and has many other proteolytic substrates besides fibrin, such as extracellular matrix components, growth factors, complement proteins and IgG [55,56]. Many pathogens, including bacteria, fungi and parasites, have been described to use plasminogen activation to their advantage [55,56]. Many bacteria recruit plasminogen to their surface through surface-exposed plasminogen-binding proteins [56]. This immobilization of plasminogen induces conformational opening of plasminogen, which enhances activation of plasminogen by host-derived activators, facilitating bacterial invasion and dissemination [55,56]. Additionally, immobilized plasminogen is protected against inhibition by the serine peptidase inhibitor α2-antiplasmin [56]. Surface binding and activation of plasminogen to plasmin by schistosome species has been extensively studied [10,57–60] and revealed several plasminogen-binding proteins. Several schistosomal proteins have been demonstrated to enhance the conversion of plasminogen to plasmin. These are enolase of *S. bovis, S. japonicum*, and *S. mansoni*, and annexin *of S. bovis*, and all require the presence of the natural activator of plasminogen, tissue-type plasminogen activator (tPA), for enhancement of plasminogen activation [10,58–60]. Although the interaction between plasminogen and schistosomes has been studied in detail, no studies have focussed on the interaction between schistosomal tegumental proteins and tPA, which is therefore the topic of **Chapter 6** of this thesis. In **Chapter 6** we have identified many tPA binding proteins of *S. mansoni* that are reported to be localized in the tegument. This would indicate binding of tPA at the host-parasite interface, which could aid in enhancement of fibrinolytic pathways by the adult parasite. Future studies are required to demonstrate binding of tPA to the outer surface of the worm. We have attempted several microscopy strategies to visualize binding of tPA to the outer surface, but without success. This could be attributed to auto-fluorescence of the adult worm, which hampered the use of fluorescent microscopy. Otherwise, it is possible that only a small amount of tPA binding to the surface is required for enhancement of fibrinolysis, which would make it difficult to detect using a microscopy approach.

As mentioned above, many pathogens, including bacteria use the fibrinolytic system to their own advantage. Two strategies can be discriminated in the way bacteria activate the fibrinolytic system: 1) the use of bacterial plasminogen activators, or 2)

plasminogen binding to the surface and use of host-derived activators. Schistosomes use the second strategy, while both *Streptococcus* and *Staphylococcus* species employ the first strategy. These bacteria produce the plasminogen activators streptokinase and staphylokinase that form a complex with plasminogen, which leads to its activation [56]. Both streptokinase and staphylokinase are used as thrombolytic treatment in patients with acute myocardial infarction or arterial thrombosis [61,62]. The second strategy, using host-derived activators for the activation of immobilized plasmin, which is used by schistosomes, is likely not suitable to be used as a thrombolytic therapy in the clinic. This strategy relies on recruitment of host protein activators of plasminogen, such as tPA, which allows plasminogen activation by pathogens when no fibrin clot is present. However, during a thrombotic event it is unlikely that this strategy will have a beneficial therapeutic effect, as the fibrin fibres present in the thrombus would form a sufficiently strong substrate for plasminogen activation and recruitment of plasminogen activators. This assumption is strengthened by the fact that, although to date many bacterial plasminogen receptors have been identified [56], none of these are currently used as thrombolytic therapies.

F. hepatica *cathepsin L peptidases*

Not only blood-dwelling parasites, such as schistosomes, interact with the haemostatic system of their host. Also blood-feeding parasites, such as *Fasciola* species encounter the blood coagulation system. In order to feed for prolonged periods on the blood of their host, many blood-feeding parasites have evolved mechanisms to prevent coagulation. Moreover, some of these have turned out to be potent antithrombotic agents, such as the leech-derived thrombin inhibitor hirudin [63]. In **Chapter 7** we have studied several cathepsin peptidases of *Fasciola hepatica* and *Schistosoma mansoni* for their anticoagulant properties. In **Chapter 7** we demonstrated that the *F. hepatica* cathepsin L peptidases (FhCL) 1, 2, and 3 degraded purified fibrinogen and that additionally FhCL1 and FhCL2 both efficiently degraded fibrin.

Haemostatic abnormalities during fascioliasis have been studied in infected cattle and sheep. The studies performed in cattle show contradicting results on the presence of coagulation changes in fascioliasis: where one study demonstrates a prolongation of PT [64], the other study reports no changes in clotting time [65]. Only a single study has examined clotting times during fascioliasis in sheep and reports an acceleration of APTT and prolonged PT and thrombin time (TT; common pathway), both in plasma of infected sheep as well as *in vitro* using excretory/secretory products of the parasite [2]. Although coagulopathy during fascioliasis has been described in several studies, the outcome varies between the studies which makes establishing the exact coagulation changes during fascioliasis difficult. It has been suggested that the observed coagulopathy is a result of both decreased production of coagulation

factors caused by liver damage and increased consumption of coagulation factors, in particular prothrombin, caused by activation of the intrinsic coagulation pathway [2]. This will result in prolongation of PT and TT, but a direct effect of parasite excretory/secretory products on fibrinogen or thrombin cannot be excluded. The suggested involvement of liver damage and coagulation factor consumption in the arise of coagulopathy is in line with the observation that all in **Chapter 7** tested peptidases are strongly inhibited by human plasma components. This is not surprising as plasma contains a large array of peptidase inhibitors [66]. Therefore it is likely that the tested cathepsin peptidases do not affect the haemostatic system of their host. Instead, we propose that they function in the *Fasciola* gut in the degradation of fibrinogen and fibrin. Secretion of these cathepsin peptidases at high concentrations in the *Fasciola* gut would allow them to locally overcome the effects of plasma peptidase inhibitors, facilitating blood-feeding of the parasite for prolonged periods. Many pathogens are known to interact with fibrinogen and/or fibrin, and many bacterial factors have been described that degrade fibrinogen or fibrin. This plays an important role in bacterial invasion and dissemination, through clearance of fibrin networks that restrict bacterial movement, but is also involved in immune evasion, as fibrin(ogen) is involved in stimulation of host immune cell activity [67,68]. Degradation of fibrinogen and fibrin by *Fasciola* cathepsin peptidases may thus also be involved in local immune modification to allow long-term survival of the parasite.

Haemostasis and parasitic helminths: future perspectives

In this thesis we have focussed on the interaction between the haemostatic system and parasitic helminths, in particular *Schistosoma* and *Fasciola* species. The blood-dwelling schistosomes have developed a broad and complex set of strategies to modify the coagulation system at different levels. The work in this thesis has revealed that adult schistosomes interact with the plasma protein VWF, an interaction that was previously only known for schistosome eggs. Additionally, we have demonstrated the presence of tPA binding proteins associated with the tegument of schistosomes, and thereby provided more insight into modification of fibrinolysis by schistosomes. As mentioned before, it is unlikely that this thesis provides a complete overview on coagulation modification by schistosomes and novel discoveries will provide further insight into the complex host-parasite relationship.

Further studies are needed to examine whether SmCB2 can be applied in disorders in which regulation of VWF functionality by ADAMTS13 is disturbed, such as thrombotic thrombocytopenic purpura. Additionally, VWF has been implied to contribute to other thrombotic diseases and complications, including stroke, myocardial infarction,

venous thrombosis, and renal and cardiovascular complications in diabetes mellitus [69]. Targeting VWF in these disorders with SmCB2 as a VWF-cleaving peptidase could provide novel preventive or therapeutic options.

The in **Chapter 7** examined cathepsin L peptidases of *Fasciola* have been extensively studied for their potential as vaccine candidates [70]. Cathepsin L peptidases are vital in host-parasite interactions of *Fasciola* and are involved in blood-feeding and immune evasion [70,71]. Their importance during infection makes these cathepsin L peptidases interesting targets for the development of novel anti-parasitic therapies. Cysteine peptidase inhibitors have been studied as novel chemotherapy against schistosomiasis. Worm burden and pathology were significantly reduced by administration of a vinyl sulfone cysteine peptidase inhibitor in a murine model of schistosomiasis mansoni [72]. Similarly, administration of cysteine peptidase inhibitors in fascioliasis may have potential as a novel anti-parasitic therapy of fascioliasis.

Conclusion

With the work described in this thesis, we have provided novel insight into the mechanisms underlying coagulopathy during schistosomiasis and the mechanisms used by helminth parasites to modify coagulation activation. Identification of new coagulation modification strategies opens new doors for the development of drugs and vaccines against these parasites. Additionally, some of these mechanisms may have applications beyond parasitic disease, as novel antithrombotic or thrombolytic agents in haemostatic disorders. Especially the identification of SmCB2 as a VWF cleaving peptidase might be applicable as a novel treatment for disorders in which regulation of VWF functionality by ADAMTS13 is disturbed, such as thrombotic thrombocytopenic purpura (TTP).

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A

APPENDICES

Nederlandse samenvatting
Curriculum vitae
List of publications
PhD portfolio
Dankwoord

Nederlandse samenvatting

Introductie

De biologische term “parasitisme” beschrijft de relatie tussen twee organismen, waarbij één organisme (de parasiet) leeft ten koste van het andere organisme (de gastheer). Hoewel zeer veel organismen een parasitaire levensstijl hebben, wordt de term “parasiet” slechts gebruikt voor (I) parasitaire eencellige eukaryoten (de protozoa), (II) parasitaire wormen (de helminthen), en (III) ectoparasieten. Dit proefschrift is gericht op de parasitaire wormen, in het bijzonder de in de bloedbaan levende platwormen behorend tot het genus *Schistosoma* en de bloed-consumerende platwormen behorend tot het genus *Fasciola*.

Beide parasitaire wormen zijn humane ziekteverwekkers en vormen voornamelijk in tropische landen een groot gezondheidsprobleem. Schistosomen zijn de veroorzakers van de tropische ziekte schistosomiasis en wereldwijd zijn meer dan 200 miljoen mensen geïnfecteerd met deze parasiet. Platwormen behorend tot het genus *Fasciola*, ook wel leverbotten genoemd, zijn de veroorzaker van fasciolose. Wereldwijd lijden minstens 2.4 miljoen mensen aan fasciolose. Daarnaast zijn leverbotten een belangrijke verwekker van parasitaire worminfecties bij vee en hebben daardoor een grote economische impact.

Beide helminthen hebben een complexe levenscyclus, die zowel een volwassen stadium in een zoogdier (de eindgastheer), als een tussenstadium in een zoetwaterslak (de tussengastheer) omvat. Infectie met schistosomen vindt plaats na contact met besmet zoet water, waarbij de *Schistosoma* larve (cercaria) door de huid naar binnen gaat en vervolgens een bloedvat binnendringt. De onvolwassen wormen (schistosomula) verplaatsen zich via de bloedbaan naar de lever, waar de volwassen wormen paren vormen. Hierna migreren de gepaarde wormen naar de bloedvaten rondom de darm (*Schistosoma mansoni* en *S. japonicum* soorten), of de vaten rondom de urineblaas (*S. haematobium* soort). De volwassen mannelijke en vrouwelijke worm blijven in deze bloedvaten gepaard aanwezig voor een periode van soms wel tientallen jaren. De symptomen van schistosomiasis worden niet zo zeer veroorzaakt door de aanwezigheid van het wormpaar, maar door de eieren die worden geproduceerd door de vrouwelijke worm. Een substantieel deel van de gelegde eieren wordt niet met de feces of urine uitgescheiden en komt vast te zitten in het leverweefsel (voornamelijk bij *S. mansoni* en *S. japonicum* infecties) of in de wand van de urineblaas (*S. haematobium*), met weefselschade en klinische symptomen tot gevolg. Wanneer uitgescheiden eieren in contact komen met zoet water, komt de larve (miracidium) uit het ei en infecteert de tussengastheer, een in de (sub)tropen levende zoetwaterslak. In de tussengastheer vermenigvuldigen en ontwikkelen de larven zich tot een voor de mens infectieuze larve (cercaria) die in het water uitgescheiden wordt. Infectie met leverbotten vindt plaats na ingestie van met ingekapselde larven (metacercariae) besmette (water)planten. In de dunne darm komen deze larven

uit hun kapsel en migreren door de darmwand en de buikholte naar de lever. Na een migratie door het leverweefsel die weefselschade en klinische symptomen veroorzaakt, komen de wormen meerdere weken na infectie aan in de galgang, waar ze volwassen worden. Volwassen leverbotten kunnen meerdere jaren aanwezig blijven in de galgang, waar ze zich voeden met bloed van hun gastheer. De eieren die de parasiet produceert, worden met de gal naar de darm vervoerd en met de feces uitgescheiden. In zoet water komt de larve (miracidium) uit het ei en infecteert de tussengastheer, een zoetwaterslak. In de tussengastheer vermenigvuldigen en ontwikkelen de larven zich tot cercariae die in het water uitgescheiden worden. De cercariae binden aan planten die aanwezig zijn in het besmette water en kapselen zichzelf in tot metacercariae die voor mens en dier infectieus zijn.

Hoewel de *Schistosoma* en *Fasciola* parasieten sterk van elkaar verschillen, hebben ze beide tijdens hun levenscyclus voor langere periode contact met het bloedstollingssysteem van hun gastheer, omdat ze zich in de bloedbaan bevinden en/of zich voeden met het bloed van hun gastheer.

Het bloedstollingssysteem van mensen bestaat uit verschillende sterk gereguleerde mechanismen die samen bloedverlies voorkomen op plekken waar de bloedvatwand beschadigd is. Daarnaast speelt het bloedstollingssysteem ook een belangrijke rol in de afweer tegen ziekteverwekkers. Beschadigingen aan de vaatwand of activatie van endotheelcellen stimuleren de binding (via von Willebrand Factor (VWF) en collageen) en de activatie en aggregatie van bloedplaatjes. Dit leidt op de plek waar de vaatwand beschadigd is tot vorming van een plaatjesprop die de beschadiging afdicht. Deze plaatjesprop is echter instabiel en moet gestabiliseerd worden tot een stabiel stolsel door de vorming van fibrinedraden (door het enzym trombine) die de plaatjes aan elkaar verbinden in een proces dat bloedstolling wordt genoemd. Bloedstolling is een sterk gereguleerde opeenvolging van enzymatische reacties waarbij diverse bloedstollingseiwitten in bloedplasma worden geactiveerd, waarna uiteindelijk trombine actief wordt en fibrinogeen kan omzetten in fibrinedraden. Deze activatie van bloedstollingseiwitten kan geïnduceerd worden door factoren op beschadigd weefsel (tissue factor route), maar ook door de aanwezigheid van een negatief geladen oppervlak (contact activatie route). Na reparatie van de beschadiging in de vaatwand moet het stolsel afgebroken worden. Dit proces heet fibrinolyse en het enzym plasmine speelt hierin de hoofdrol door klieving van de fibrinedraden die het stolsel stabiliseren.

Contact tussen een parasitaire worm en het bloed van de gastheer zou op verschillende manieren kunnen leiden tot activatie van het bloedstollingssysteem van de gastheer. De aanwezigheid van de parasiet in de bloedbaan en binding aan de vaatwand kan leiden tot activatie of beschadiging van de vaatwand en verstoring van de bloedstroom, wat een sterke stimulus vormt voor de activatie van bloedplaatjes en

stollingseiwitten in plasma. Daarnaast vormt het oppervlak van de parasiet een sterke stimulus voor activatie van de in plasma aanwezige stollingseiwitten via de contact-activatie. Echter, zowel schistosomen als leverbotten overleven lange perioden van blootstelling aan het bloedstollingssysteem van hun gastheer. Het is daarom waarschijnlijk dat de parasieten strategieën ontwikkeld hebben om de activatie van het bloedstollingssysteem in te perken. Het doel van het onderzoek beschreven in dit proefschrift is daarom om: (I) een overzicht te geven van de tot op heden bekende mechanismen die door schistosomen gebruikt worden voor de beïnvloeding van het bloedstollingssysteem van de gastheer, (II) inzicht te krijgen in de directe effecten van *Schistosoma* parasieten op de bloedstolling van de gastheer tijdens infecties met *S. haematobium* waarin leverschade niet opgetreden is, en (III) om tot nu toe onbekende strategieën voor de beïnvloeding van het bloedstollingssysteem door schistosomen en leverbotten te identificeren.

Effecten van schistosomen op de bloedstolling: huidige status

Hoofdstuk 2 van dit proefschrift beschrijft de grote verscheidenheid aan mechanismen die schistosomen gebruiken om de verschillende onderdelen van het bloedstollingssysteem van de gastheer te beïnvloeden. Schistosomen gebruiken hiervoor parasiet-specifieke mechanismen, maar bootsen ook mechanismen van de gastheer na of maken gebruik van mechanismen van de gastheer voor regulering van bloedstolling. Schistosomen hebben mechanismen die de activatie en aggregatie van bloedplaatjes remmen en daarmee de vorming van een plaatjesprop inperken. Daarnaast produceren schistosomen remmers van stolling en produceren ze moleculen die de mechanismen van de gastheer voor regulering van stolling activeren. Tot slot zijn schistosomen in staat het fibrinolyse-enzym plasmine te activeren op hun oppervlak, waardoor ze de afbraak van gevormde stolsels aan hun oppervlak kunnen stimuleren.

De grote variatie aan mechanismen beschreven in **Hoofdstuk 2** die door de parasiet worden gebruikt om bloedstolling te remmen, laat zien dat de parasiet een opvallend sterk vermogen heeft om het bloedstollingssysteem van zijn gastheer op alle verschillende niveaus te beïnvloeden, wat activatie van bloedstolling rondom de parasiet voorkomt.

Afwijkingen in bloedstolling tijdens *Schistosoma* infectie: wat leren we van *S. haematobium* infecties?

Het vermogen van schistosomen om het bloedstollingssysteem van zijn gastheer te beïnvloeden, leidt tot de vraag of deze mechanismen alleen lokaal of ook systemisch de bloedstolling van de gastheer beïnvloeden. Er is veel onderzoek gedaan naar afwijkingen in de bloedstolling tijdens infecties met *S. mansoni* en *S. japonicum*, waarbij

tijdens langdurige infecties afwijkingen aan de bloedstolling op systemisch niveau aanwezig zijn. Hieruit kan echter niet geconcludeerd worden dat dit een direct effect is van beïnvloeding van bloedstolling door de volwassen worm. Chronische infectie met *S. mansoni* of *S. japonicum* leidt namelijk ook tot ernstige beschadiging van de lever door *Schistosoma* eieren die vast komen te zitten in het leverweefsel. De lever speelt een belangrijke rol in de bloedstolling, aangezien zeer veel bloedstollingseiwitten geproduceerd worden in de lever. Leveraandoeningen gaan daarom vaak hand in hand met afwijkingen in de bloedstolling. De afwijkingen in de bloedstolling die tijdens chronische infectie met *S. mansoni* of *S. japonicum* detecteerbaar zijn, kunnen dus zowel een direct effect van de volwassen parasiet op systemische bloedstolling, als effecten van leverschade reflecteren. Om hier onderscheid in te maken hebben we in **Hoofdstuk 3** de bloedstolling bestudeerd in een groep schoolkinderen uit Gabon die geïnfecteerd zijn met *S. haematobium*. Aangezien deze parasiet zich bevindt in de bloedvaten rondom de urine-blaas en niet of nauwelijks in de vaten rondom de darm, komt alleen tijdens zeer ernstige infecties met deze parasiet leverschade voor. Dit maakt het dus mogelijk om de effecten van de volwassen worm op systemische bloedstolling te bekijken in de afwezigheid van leverschade. Uit de resultaten van deze studie blijkt dat schistosomen ontstekings-gemedieerde activatie van endotheelcellen veroorzaken, wat wordt gekenmerkt door verhoogde niveaus van de eiwitten VWF en osteoprotegerin in plasma. Deze endotheelcelactivatie leidt echter niet tot systemische activatie van bloedstolling of fibrinolyse mechanismen in deze patiënten. Dit suggereert dat de aanwezigheid van de volwassen parasiet niet leidt tot systemische afwijkingen in de bloedstolling en dat de systemische afwijkingen in bloedstolling die worden gezien tijdens infectie met *S. mansoni* of *S. japonicum* dus zeer waarschijnlijk veroorzaakt worden door leverschade als gevolg van eieren die in het leverweefsel vast komen te zitten.

Beïnvloeding van de hemostase door helminthen: nieuwe mechanismen

Alternatieve manieren om von Willebrand Factor activiteit te reguleren

De observatie dat de concentratie van VWF in plasma verhoogd is tijdens infectie met schistosomen, zonder dat dit leidt tot activatie van stolling suggereert dat schistosomen mechanismen hebben om deze verhoogde uitscheiding van VWF door de vaatwand tegen te gaan en zo binding en activatie van bloedplaatjes te voorkomen. Normaal wordt de activiteit van VWF gereguleerd door het VWF-klievende peptidase ADAMTS13, wat altijd aanwezig is in plasma. Het is echter mogelijk dat schistosomen hun eigen mechanismen hebben ontwikkeld om de activiteit van VWF te reguleren. In **Hoofdstuk 4** is daarom het onderzoek beschreven waarin onderzocht is of volwassen schistosomen effecten hebben op VWF, wat heeft geresulteerd in de ontdekking van een VWF-klievend cysteïne peptidase

dat geproduceerd wordt door *S. mansoni*. We konden aantonen dat dit peptidase SmCB2 is, een cathepsine B peptidase uit het tegument van adulte *Schistosoma* wormen. Deze ontdekking geeft aan dat *S. mansoni* in staat is de activiteit van VWF te reguleren om zo activatie van bloedstolling te voorkomen. Dit peptidase zou mogelijk toegepast kunnen worden in ziekten waar de activiteit van VWF niet goed gereguleerd is, zoals trombotische trombocytopenische purpura (TTP), wat wordt gekenmerkt door een verlaagde activiteit van het VWF-klievende peptidase ADAMTS13. Het onderzoek beschreven in **Hoofstuk 4** heeft namelijk ook aangetoond dat het *S. mansoni* VWF-klievende peptidase niet wordt geremd door plasmafactoren specifiek aanwezig in het plasma van TTP patiënten.
Het feit dat *S. mansoni* in staat is om op een alternatieve manier VWF te reguleren, spoorde ons aan om te onderzoeken of er ook alternatieve routes voor regulatie van VWF activiteit bestaan in de mens (**Hoofdstuk 5**). Eerder is gevonden dat het fibrinolytische enzym plasmine in staat is VWF af te breken en is gesuggereerd dat plasmine een back-up mechanisme zou kunnen zijn voor het VWF-regulerende enzym ADAMTS13 dat vermindert actief is in TTP patiënten. Er zijn echter ook negatieve effecten van plasmine op ADAMTS13 beschreven, aangezien verschillende studies beschrijven dat ADAMTS13 wordt afgebroken door plasmine, wat mogelijk een risico zou kunnen vormen in TTP patiënten. De resultaten beschreven in **Hoofdstuk 5** van dit proefschrift laten echter zien dat klieving van ADAMTS13 door plasmine de activiteit van het ADAMTS13 enzym verhoogt en dus geen negatief effect heeft op het ADAMTS13 enzym. Deze resultaten laten voor het eerst enzymatische regulatie van ADAMTS13 activiteit zien. Tot op heden werd namelijk aangenomen dat de activiteit van ADAMTS13 gereguleerd wordt door conformatieveranderingen in VWF, onder invloed van de snelheid van de bloedstroom, en in ADAMTS13 zelf, na binding aan VWF. Plasmine kan dus een alternatief mechanisme vormen om lokaal ADAMTS13 activatie, en daarmee dus indirect ook de activiteit van VWF, te reguleren en zo lokaal bloedstolling te beïnvloeden.

Effecten van Schistosoma *en* Fasciola *op fibrinogeen, fibrine en de fibrinolyse*

Plasmine vormt ook het belangrijkste enzym in het fibrinolytische systeem dat verantwoordelijk is voor de afbraak van stolsels. Veel studies hebben aangetoond dat schistosomen in staat zijn om plasminogeen te binden aan hun oppervlak en de activatie tot plasmine te stimuleren. Al deze studies tonen echter ook aan dat dit alleen gebeurt in de aanwezigheid van tissue-type plasminogeen activator (tPA) dat plasminogeen om kan zetten in plasmine. Het is echter onduidelijk of tPA ook kan binden aan het oppervlak van de worm. Daarom hebben we onderzocht of er tPA-bindende eiwitten aanwezig zijn op het oppervlak van *S. mansoni* parasieten (**Hoofdstuk 6**). Dit onderzoek toont aan dat de parasiet een groot aantal tPA bindende eiwitten bevat, die ten dele ook aan het oppervlak van de parasiet voorkomen. Dit suggereert dat binding van zowel plasminogeen als tPA op het oppervlak van de worm zorgt voor lokale activatie van plasmine en een fibrinolytische omgeving creëert rondom de

worm. Tot slot is onderzocht of gezuiverde cathepsine peptidases van *S. mansoni* en *F. hepatica* ook direct fibrinogeen en fibrinedraden af kunnen breken (**Hoofdstuk 7**). Hoewel de *S. mansoni* peptidases fibrinogeen en fibrine niet direct konden afbreken, konden drie verschillende cathepsine L peptidases van *F. hepatica* (FhCL1, FhCL2, en FhCL3) dit wel. Hoewel fibrinogeen en fibrine een substraat vormen voor bepaalde cathepsine L peptidases van *F. hepatica*, betekent dit niet direct dat deze peptidases ook een effect hebben op het bloedstollingssysteem van de gastheer. Uit het onderzoek beschreven in **Hoofdstuk 7** blijkt namelijk ook dat deze peptidases sterk geremd worden door componenten aanwezig in humaan plasma wat suggereert dat de peptidases waarschijnlijk de bloedstolling van de gastheer niet beïnvloeden. Veel waarschijnlijker is dat deze peptidases in de darm van de parasiet betrokken zijn bij de afbraak van fibrinogeen en fibrine, waardoor het door de parasiet opgenomen bloed niet stolt in de darm van de parasiet wat de consumptie van bloed door de parasiet mogelijk maakt.

Conclusie

Het onderzoek beschreven in dit proefschrift geeft nieuwe inzichten in de mechanismen die parasitaire wormen gebruiken om lokaal de bloedstolling van de gastheer te beïnvloeden. Daarnaast heeft ons onderzoek naar afwijkingen in de bloedstolling tijdens infecties met *S. haematobium* ook meer inzicht gegeven in de directe effecten van de volwassen parasiet op het bloedstollingssysteem van de gastheer. De identificatie van nieuwe mechanismen die door parasitaire wormen worden gebruikt om bloedstolling te beïnvloeden, kunnen leiden tot de ontwikkeling van nieuwe medicijnen voor de behandeling van infecties met deze parasieten. Daarnaast kunnen sommige van deze mechanismen mogelijk toegepast worden als nieuwe antistollingsmiddelen.

Curriculum vitae

Mirjam Maaike Mebius was born on the 7th of July 1989 in Amersfoort (the Netherlands). She followed her secondary education (VWO) at the Willem de Zwijger school (Schoonhoven), which she successfully completed in 2007. Mirjam focussed her education in the areas of health and science by completing the profiles "Natuur & Gezondheid" and "Natuur & Techniek". She continued her education at Utrecht University and obtained her Bachelor degree (*cum laude*) in Biomedical Sciences in 2010. As a result of her interest in infectious diseases and the interplay between pathogens and their host, she started her Master study "Infection and Immunity" at Utrecht University. During her Master education she completed a 9-month internship focused at infectious diseases in the group of Prof. dr. Jos van Strijp (department of Medical Microbiology, University Medical Centre Utrecht) and a 7-month internship focused on immunology in the group Prof. dr. Leonie Taams (Centre for Molecular and Cellular Biology of Inflammation, King's College London, United Kingdom). In the group of Prof. dr. Jos van Strijp she studied the use of a phage display library displaying *Staphylocuccus aureus* secreted proteins as a novel tool in the identification of immune modulatory proteins of *S. aureus* under supervision of Dr. Cindy Fevre and Dr. Pieter-Jan Haas. In the group of Prof. dr. Leonie Taams she was involved in two research projects. She studied the involvement of the Prickle protein, a negative regulator of Wnt signalling, in immune responses in *Drosophila melanogaster* and in human monocytes under supervision of Prof. dr. Leonie Taams and Dr. Marc Dionne. Additionally, she studied the *in vitro* effects of TNFi drugs on monocyte viability under supervision of Prof. dr. Leonie Taams. She wrote her Master thesis under supervision of Prof. dr. Lodewijk Tielens and Dr. Jaap van Hellemond (department of Medical Microbiology and Infectious Diseases, Erasmus MC) on the strategies employed by parasitic worms of the *Schistosoma* genus to ensure survival in the human bloodstream and in 2012 she obtained her Master degree (*cum laude*). Her interest in host-pathogen interactions and her enthusiasm for the subject of her Master thesis, resulted in the start of her PhD-training at the department of Medical Microbiology and Infectious Diseases (Erasmus MC, Prof. dr. Lodewijk Tielens and Dr. Jaap van Hellemond) in close collaboration with the Laboratory of Clinical Chemistry and Hematology (University Medical Centre Utrecht, Prof. dr. Flip de Groot and Dr. Rolf Urbanus). The results of the research she performed during her PhD-training are presented in this thesis. In 2017 she continued her career as post-doctoral researcher at Kinetic Evaluation Instruments (Utrecht), where she was involved in the development of a novel diagnostic test for the detection of tuberculosis in humans. Mirjam currently works as senior research associate at the department of Antibody Research and Technology at Genmab (Utrecht), where she is involved in the development of novel antibody therapeutics for the treatment of cancer.

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‡Authors contributed equally

PhD portfolio

Name candidate:	Mirjam Maaike Mebius
Institute:	Erasmus University Medical Center Rotterdam
Department:	Medical Microbiology and Infectious Diseases
PhD period:	2012-2017
Research school:	Molecular Medicine (MolMed) postgraduate school
Promotors:	Prof. dr. J.W. Mouton †
	Prof. dr. A.G.M. Tielens
	Prof. dr. Ph.G. de Groot
Copromotors:	Dr. J.J. van Hellemond
	Dr. R.T. Urbanus

Courses	Year
Handling laboratory animals, article 9, University Medical Center Amsterdam, Amsterdam	2012
Research management for PhD-students, MolMed postgraduate school, Rotterdam	2014
Dutch Society for Thrombosis and Haemostasis – Bleeding Disorders, Koudekerke	2014
Research integrity, MolMed postgraduate school, Rotterdam	2014
Photoshop and illustrator CS6 workshop for PhD-students and other researchers, MolMed postgraduate school, Rotterdam	2014
Dutch Society for Thrombosis and Haemostasis - Venous Thrombosis, Koudekerke	2015
Biomedical research techniques, MolMed postgraduate school, Rotterdam	2015
“Mondeling presenteren”, Utrecht University, Utrecht	2016
Course presenting skills for scientists, MolMed postgraduate school, Rotterdam	2016
Dutch Society for Thrombosis and Haemostasis – Arterial Thrombosis, Den Haag	2016
“Netwerken werkt!”, Erasmus University Medical Center Rotterdam, Rotterdam	2016
Workshop LinkedIn, Erasmus University Medical Center Rotterdam, Rotterdam	2017

(Inter)national conferences and seminars	Year
Centre of Infectious Diseases symposium: Vaccines and Vaccination, Leiden	2012
Joint Netherlands Society for Parasitology/Belgian Society for Parasitology and Protistology scientific meeting, Antwerpen	2012
Medical Microbiology and Infectious Diseases department research day, Rotterdam (poster presentation)	2012
Netherlands Society for Parasitology autumn meeting, Rotterdam	2013
18th Molecular Medicine day, Rotterdam	2014
Dutch Society for Thrombosis and Haemostasis symposium, Koudekerke	2014
TTP patient meeting, Amsterdam	2014
Molecular and Cellular Biology of Helminth Parasites VIII, Hydra, Greece (oral presentation)	2014
"Het complete plaatje" Farewell symposium Prof. dr. Ph.G. (Flip) de Groot, Utrecht	2014
Netherlands Society for Parasitology autumn meeting, Rotterdam	2014
"Golden Oldies" Farewell symposium Prof. dr. H.A. (Henri) Verbrugh, Rotterdam	2015
19th Molecular Medicine day, Rotterdam (poster presentation)	2015
Dutch Society for Thrombosis and Haemostasis symposium, Koudekerke	2015
3rd annual SURE Symposium "THE NEXT STEP: Career Perspectives for Young Biomedical Scientists", Rotterdam	2015
Netherlands Society for Parasitology spring meeting, Utrecht (oral presentation)	2015
Netherlands Society for Parasitology autumn meeting, Rotterdam	2015
20th Molecular Medicine day, Rotterdam (poster presentation)	2016
Joint Netherlands Society for Parasitology/Belgian Society for Parasitology and Protistology spring meeting, Rotterdam (poster presentation)	2016
European Congress on Thrombosis and Haemostasis, Den Haag (oral and poster presentation)	2016

Teaching	Year
Supervision of Master internship	2015-2016
Supervision of 2nd year medical students "VO Infectieziekten"	2015-2016

Grants	Year
Netherlands organization for scientific research (NWO)/Erasmus Molecular Medicine (MolMed) postgraduate school research grant	2014
Netherlands Society for Parasitology travel grant	2014
Erasmus Trustfonds travel grant	2014

Blood-dwelling or blood-feeding parasitic helminths, such as *Schistosoma* and *Fasciola* species, interact for prolonged periods with the haemostatic system of their host. As a result of their blood-feeding behavior or presence in the veins, these parasites are expected to be potent activators of the haemostatic system. However, the longevity of parasites in their host implies that they have developed strategies to modify activation of the haemostatic system to survive in continuing interaction with their human host. In this thesis, the author discusses the complex interactions between host haemostasis and parasitic helminths. The results of the studies provide novel insight into mechanisms underlying coagulopathy during schistosomiasis and the strategies used by helminth parasites to modify coagulation activation. Identification of new strategies for coagulation modification by *Schistosoma* and *Fasciola* species may have implications beyond parasitic disease, such as in the development of novel antithrombotic or thrombolytic agents in haemostatic disorders.