

HAEMOSTASIS

AND

VIRUS INFECTIONS

**- EPIDEMIOLOGY,
PATHOGENESIS &
PREVENTION -**

Marco Goeijenbier

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

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Viral infections and mechanisms of thrombosis and bleeding

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J. Med. Vir. 2012, 84(10):1680-96

Ebola Virus Disease; a review on epidemiology, symptoms, treatment and pathogenesis

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Virus infections and mechanisms of thrombosis and bleeding

Viral infections of any kind can influence the haemostatic balance. This means that during, or shortly after infection with certain viruses the ability of blood to clot in humans can be drastically changed which can lead to both thrombotic as well as haemorrhagic complications. Direct or indirect activation of the endothelium by viruses (or other pathogens) may result in alterations in the coagulation- and the fibrinolytic systems (1). Normally, coagulation is a balance between procoagulant and (natural) anticoagulant mechanisms and actually it seems that a well regulated activation of coagulation is part of the host's defence against infectious agents (2). However, the inflammation caused by certain viral infections may lead to such alterations in coagulation resulting in an imbalance between the pro- and anticoagulant state. The clinical picture of altered coagulation in several viral infections manifests itself in mild or even severe bleeding (haemorrhage), thrombosis, or both. In severe cases this may even lead to disseminated intravascular coagulation with the formation of micro vascular thrombi in various organs (3). Disseminated intravascular coagulation contributes to multiple organ failure and is associated with high mortality in both bacterial and nonbacterial diseases (3;4). It is not yet clear why some viruses cause haemorrhage (e.g. the viral haemorrhagic fevers like hantavirus and Ebola), others are associated with thrombosis (e.g. cytomegalovirus and influenza) and others show both (e.g. varicella zoster virus) (5-8). In addition, the bleeding complications caused by viral haemorrhagic fever pathogens vary in severity, such as more minor bleeding complications in dengue and more severe bleeding seen in patients infected with Crimeon Congo Haemorrhagic fever virus or one of the Old-World hantaviruses. Both bleeding and thrombotic complications during or shortly after viral infection are associated with a worse outcome of disease, while for most of the viral infections no targeted therapy is available and only supportive care can be provided. Therefore, a better understanding is needed of the pathogenesis of bleeding and thrombotic complications due to viral infections.

Principles of haemostasis, coagulation, and fibrinolysis: general aspects

One of the most important functions of the endothelial cell layer is the maintenance of haemostasis, a physiologic mechanism that maintains blood in a fluid state within the vasculature and responds with coagulation at time of injury to prevent exsanguination (9). For this to work properly, a perfect balance between pro- and anti-coagulant factors is necessary. The formation of a blood clot is a well-regulated process comprising three elements: 1) primary haemostasis, 2) secondary haemostasis/coagulation, and 3) fibrinolysis (Figure 1) (10). The onset of clotting arises when the vasculature is damaged and blood is exposed to platelet activating- and pro-coagulant factors. The first response, primary haemostasis, is the adherence and aggregation of activated platelets to the side of injury, due to Von Willebrand factor (VWF). Von Willebrand factor is a very sensitive marker of endothelial cell activation and is a large

glycoprotein synthesized in the vascular endothelium. Endothelial cells store VWF in the Weibel-Palade bodies in the form of unusually large vWF multimers. The function of vWF comprises adhesion of platelets to exposed subendothelium, forming the basis for the haemostatic plug. At the site of injury activated platelets provide cell-surface phospholipids to further facilitate secondary haemostasis, the formation of fibrin strands, which stabilise the platelet plug. Coagulation results from a series of linked coagulation protease-zymogen reactions, ultimately resulting in the formation of fibrin. Tissue factor is the main initiator of the coagulation cascade, which is localised in the subendothelium, but also on non-circulating leukocytes and possibly on platelets. Thrombin generation is induced by the assembly of the tissue factor-factor VIIa complex. Thrombin is able to convert fibrinogen into (insoluble) fibrin. Coagulation is regulated by different inhibitory mechanisms. A first mechanism is made up of the circulating inhibitors of blood coagulation: antithrombin and heparin cofactor II (both inhibitors of thrombin), and tissue factor pathway inhibitor. Two other circulating inhibitors of blood coagulation are protein C and protein S (the latter of which is a cofactor for the proper functioning of activated protein C). A second inhibitory mechanism consists of the endothelium-bound modulators heparin sulphate and thrombomodulin, which facilitate the inhibitory activity of antithrombin and the activation of protein C, respectively. The third element, the fibrinolytic system, is necessary to degrade the formed fibrin strands. This system is initiated by tissue plasminogen activators and urokinase after their synthesis by, and release from, endothelial cells. These activators initiate the conversion of plasminogen to plasmin, which hydrolyses polymerised fibrin strands into soluble fibrin degradation products, thus degrading the fibrin clot. The activity of the fibrinolytic system is, among other things, regulated by plasminogen activator inhibitor type I, which may greatly increase during acute phase reactions. Thrombin-activatable fibrinolysis inhibitor is also an inhibitor of fibrinolysis and is activated, as suggested by the name, by thrombin. Fibrinolysis may be activated primarily – and thus independently of the activation of the coagulation cascade – or secondarily, in response to fibrin formation.

Procoagulant/prothrombotic changes

Procoagulant and prothrombotic are two terms used in literature to indicate the same mechanism; a tendency to develop thrombosis. In this thesis the term procoagulant is used to refer to a change in coagulation markers determined in the laboratory, while a prothrombotic state indicates a clinical risk situation. In other words the risk for the development of thrombosis is increased in a patient due to any factor (immobility, malignancy). Generally speaking, in viral infections several procoagulant markers are elevated, indicating that the coagulation system is activated.

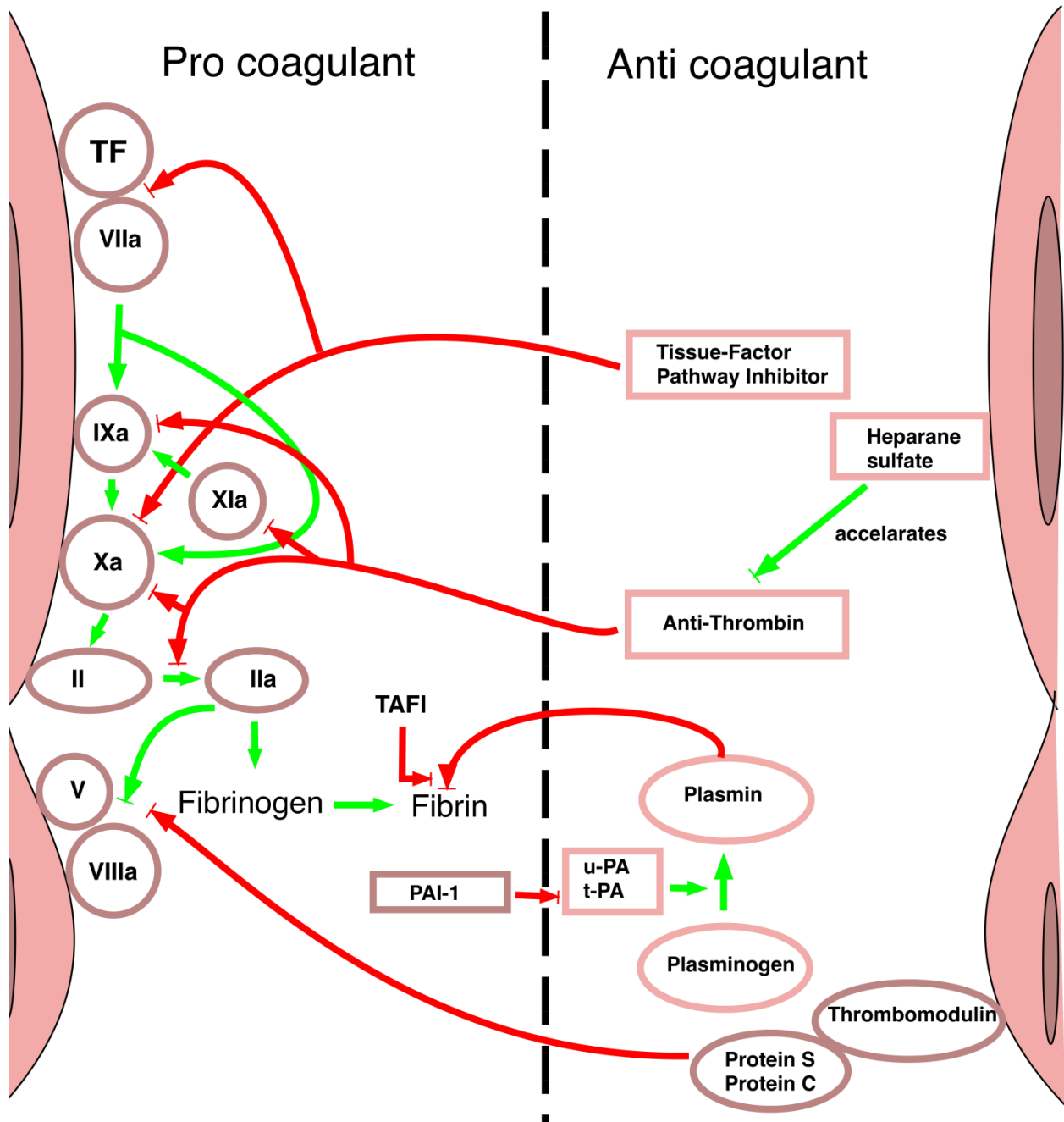


Figure 1. Simplified model of the coagulation cascade. Coagulation proteins are given in roman numerals. TF = tissue factor, PAI-1 = plasminogen activator inhibitor type 1, u-PA = urokinase-type plasminogen activator, t-PA = tissue-type plasminogen activator, TAFI = thrombin activatable fibrinolysis inhibitor

A clear procoagulant state can be defined by a number of alterations in the blood. Firstly, increased levels of clotting factors (e.g. factor VIII, factor XI), soluble tissue factor and von Willebrand factor. Secondly, deficiency of the regulatory [anti-coagulant] proteins, e.g. protein C, protein S, antithrombin, and tissue factor pathway inhibitor. Furthermore, markers of

thrombin generation (prothrombin fragment 1+2 and thrombin-antithrombin complexes), platelet activation, fibrin degradation and fibrinolysis (e.g. D-dimer and plasmin- α 2-antiplasmin complexes) can also be increased.

The effect of inflammation on coagulation

An increasing amount of evidence suggests the existence of an extensive correlation between inflammation and coagulation, whereby inflammation not only leads to coagulation, but coagulation also affects inflammatory activity (2;11-13). Inflammation impacts the initiation, propagation and inhibitory phases of blood coagulation (2). Many studies have been carried out on the influence of inflammation, in particular the (pro)inflammatory cytokines on the coagulation system. The activation of the coagulation cascade during inflammation is the result of the stimulation of coagulant synthesis, the decreased synthesis of anti-coagulants and the suppression of fibrinolysis (14). This is the net result of an increased expression of tissue factor leading to the activation of the extrinsic coagulation pathway, together with the down regulation of activated protein C and the inhibition of fibrinolysis (15). Pro-inflammatory cytokines like IL-6, IL-1, IL-12 and TNF- α are known to increase the production and subsequent expression on the endothelial cell surface of VWF, which may result in platelet activation. They upregulate tissue factor expression via the activation of protease-activated receptors, on monocytes, and endothelial cells (2;11;16). Furthermore, the generation of activated protein C is also impaired, due to a lowered expression of thrombomodulin on the surface of endothelial cells, which acts as a cofactor in the thrombin-mediated activation of protein C. While on the one hand anticoagulants are decreased, on the other hand there is an upregulation of plasminogen activator inhibitor-1, which is able to block the activation of plasminogen, thus creating a downregulation of the breakdown of fibrin clots (2;16). The generation of thrombin, as a net result, is generally increased due to inflammation, all of which results in a procoagulant state of the clotting cascade (17).

Viral infection and haemorrhage

Viruses that most severely affect coagulation are collectively categorized as the viral haemorrhagic fever pathogens. Viral haemorrhagic fever (VHF) is a syndrome characterized by the hallmark symptoms of (high) fever, bleeding in various organs and complicated by multi-organ failure (MOF) and eventually shock. The pathogens associated with this severe clinical condition in humans originate from different families like the *Filoviridae* (Ebola virus and Marburg virus), *Bunyaviridae* (hantavirus, Crimeon-Congo haemorrhagic fever (CCHF) and Rift Valley fever), *Flaviviridae* (dengue, yellow fever) and the family of the *Arenaviridae* (Lassa and South American haemorrhagic fever). Infection with one specific VHF pathogen may result in a different clinical course compared to another, but all seem to have in common that the endothelium is activated and dysfunctional. Endothelial stress in patients who develop VHF are

at first characterized by (mild) hypotension, conjunctival vasodilation and flushing of the skin in the early phase of the disease (18). As the disease progresses endothelial dysfunction becomes more prominent and may result in capillary leakage, fluid effusion in body cavities, haemorrhage and eventually shock. The number of patients that develop haemorrhage and/or shock differs greatly within the VHF pathogens. For instance in Lassa fever, shock is relatively common, while bleeding complications are quite rare. On the other hand in patients with CCHF bleeding is quite prominent (19). In VHF bleeding often occurs from various mucous membranes together with easy bruising and persistent bleeding after venapuncture. Massive bleeding may occur in the gastro-intestinal tract and/or intra-cerebrally (Kortepeter et al, 2011). These bleeding complications are often observed in severe forms of infection and they correlate with the case fatality rate. Bleeding is thought to be the consequence of a multifactorial process of which an imbalanced coagulation cascade sometimes results in disseminated intravascular coagulation, as seen in hantavirus-, Ebola-, Marburg-, Crimean-Congo haemorrhagic fever- and dengue virus infections (5). Multiple mechanisms have been suggested in literature to explain haemorrhage and plasma leakage in VHF. Of these most generally accepted are those suggesting direct endothelial injury, platelet aggregation and consumption, liver damage, cytokine storm and (over)activation of the coagulation system (20). However, in many other viral infections that do not cause haemorrhage the coagulation system is also activated, thrombocytopenia occurs and cytokines are produced up to comparable high levels. Thus far the pathophysiologic basis for the occurrence of haemorrhage and shock in this distinct group of VHF pathogens is poorly understood.

Viral infection and thrombosis

Where VHF pathogens, and in severe cases some herpes and influenza viruses (varicella zoster virus, pandemic H1N1), are able to cause bleeding during the course of infection, many other viruses are associated with the occlusion of a vessel; thrombosis. For instance, many respiratory tract infections increase the risk of deep venous thrombosis and possibly pulmonary embolism too (21). Also multiple herpes viruses have been associated with thrombosis of any kind both in human and animal models (22). In humans, most reports describe thrombosis during CMV infection in immunocompromised patients (23-26), but also immune competent individuals may develop (most often deep vein) thrombosis during CMV infection (6;27-29). However, many of the reported cases had known additional procoagulant risk factors, such as a protein C deficiency, factor V Leiden mutation or a heterozygous prothrombin G20210A mutation (30-32). Chronic infections like hepatitis B, C and HIV are also notorious risk factors for the development of thrombosis. Both venous- and arterial thrombosis occur far more often in patients with these chronic infections compared to healthy controls. For instance the overall risk of venous thrombotic disease in HIV-infected patients is estimated to be between two and ten times as high as it is in healthy individuals (33;34). Furthermore, the treatment with first

and second generation protease inhibitors is linked to venous as well as arterial thrombotic events (34;35). The mechanisms behind these procoagulant changes have been studied for some of the viruses mentioned. In general there seems to be a central role for the vascular endothelium, which under the pressure of viral infection, shifts to a more procoagulant state. This procoagulant state is characterized by: “the inhibition of anticoagulant/antithrombotic properties, the induction of procoagulant properties of the endothelium by changing the phospholipid exposure resulting in enhanced thrombin generation and secretion of VWF and an increase in binding sites for inflammatory cells, which leads to a further procoagulant shift of the endothelial cell surface (36-39). The exact mechanism by which these viruses activate or inhibit the production of these pro- and anti-coagulant proteins is not clear yet.

Ebola virus disease

Ebola virus and the closely related Marburg virus are often typed as textbook examples of viral haemorrhagic fever pathogens. *Ebolaviruses* enter the human body via mucosal surfaces, abrasions and injuries in the skin or by direct parental transmission. Infection through intact skin is considered unlikely, although not excluded. The virus has been successfully isolated from skin (biopsy) and body fluids (40). Several laboratory associated infections have been reported the past decades, often after needle accidents or direct contact with infectious materials (41). The route of transmission seems to affect the disease outcome; in the early EBOV outbreak in 1976, case fatality rate (CFR) after transmission by injection was 100% versus 80% in contact exposure cases (42). This has been confirmed in a non-human primate model, showing faster disease progression in animals infected via injection versus those who received an aerosol challenge (43). Due to the high CFR in Ebola virus disease (EVD) and the potential threat for use of EBOV as a biodefense weapon, the pathogenesis of EVD has been relatively well studied during the past 15 years (44). Most studies have been performed in rodent-, guinea pig-, primate- and *in vitro* models. Upon entry, EBOV has proven to be able to infect numerous cell types. Post mortem studies of patients and experimentally infected animals showed infection of immune cells (macrophages, monocytes and dendritic cells), epithelial- and endothelial cells, fibroblasts, hepatocytes and adrenal gland tissue (45). Replication in infected cells is very efficient resulting in a rapid and high peak viremia (45). Furthermore, death of infected cells has been hypothesized to play an important role in the signs and symptoms seen in EVD patients, for instance the decreased ability of the immune system to respond to the infection due to apoptosis of infected lymphocytes or a decreased production of clotting factor due to the loss of hepatocytes (42). Studies addressing the mechanism behind coagulation abnormalities first showed that haemorrhage was most likely not the direct effect of endothelial cell infection, followed by cytolysis (46). A more likely explanation seems to be an overexpression of tissue factor in monocytes/macrophages resulting in (over)activation of the extrinsic pathway of coagulation followed by a consumptive coagulopathy and eventually a disseminated

intravascular coagulation (47). Furthermore antibody enhancement has been hypothesized to play a role in the later phase of the EVD course (48). Although data on this theory is still limited, antibody dependent enhancement seems to enhance infectivity of the virus *in vitro* not only for EBOV but also the closely related Marburg virus (48;49). However, due to the high CFR seen in EVD and the fact that in many patients antibody response is lowered or even absent, the mechanism of antibody enhancement does not seem clinically relevant. However, antibody enhancement has been hypothesized to play an important role in the development of dengue haemorrhagic fever (50;51) and therefore might be of interest. Interesting data about EVD pathogenesis comes from asymptomatic cases and EVD patients that survived infection. A cluster of asymptomatic infections has been described after EBOV infection. Of these 24 contacts, 11 were asymptotically infected and developed an IgM and IgG response plus a mild viremia between day 7 (first day of sampling) and day 16 (52). Although further research in mechanisms behind the haemorrhage and endothelial dysfunction seen in Ebola virus disease is of great interest bio-safety issues complicate widespread *in vitro* and *in vivo* studies.

Hantavirus infection

In regards of endothelial cell dysfunction and coagulation disturbances pathogenic hantaviruses are most interesting to study. First of all because these negative stranded RNA viruses seem to directly infect endothelial cells at an early stage of disease, in contrast to Ebola viruses discussed in the paragraph above. Furthermore, viral effects on endothelial cell function most likely contribute to the characteristic clinical features seen in hantavirus associated disease (53). Hantaviruses circulating in Europe and Asia are associated with haemorrhagic fever and renal syndrome (HFRS) (54;55). Hallmark symptoms of HFRS are fever, acute renal failure and alterations in haemostasis, ranging from mild thrombocytopenia to disseminated intravascular coagulation (56). At first, less severe bleeding complications like epistaxis, conjunctival bleeding, haematuria, petechiae and mucosal bleeding occur, while in a later phase gastrointestinal, intra-cerebral and pleural bleedings are reported in severe HFRS. Less common haemorrhagic events are right atrial haemorrhage (57), spleen haemorrhage (58) and pituitary gland haemorrhage resulting in endocrinal disturbance or even panhypopituitarism (59;60). Hantaviruses circulating in North and South America can cause Hantavirus Cardio-Pulmonary Syndrome (HCPS), resulting in acute respiratory distress and cardiac failure. However, bleeding disorders play a less significant role in the pathology of this disease. Clinical studies focusing on alterations in primary and secondary haemostasis during hantavirus disease showed thrombocytopenia in both HFRS and HCPS, a decreased plasma activity of coagulation factors II, V, VIII, IX and X in acute HFRS patients, prolongation of the prothrombin and activated partial thromboplastin time, increased thrombin generation and D-dimer levels and a decrease in ADAMTS13 activity in acute Puumala hantavirus infected patients (61-64). Furthermore, multiple *in vitro* studies investigated the role of endothelial cells in hantavirus infection and

most important findings from these experiments are summarized in Figure 2. Two very different processes seem to contribute to the activation of endothelial cells and subsequent activation of coagulation in hantavirus disease. At first, macrophages (MO) and dendritic cells (DC) induce pro-inflammatory cytokines, which evoke a changing phenotype of the endothelium towards a pro-adhesive (for immune cells) and pro-coagulant response during inflammation as discussed above. Activation of macrophages and platelets further promote the procoagulant shift. The infection of endothelial cells by hantaviruses leads to presentation of the virus glycoproteins on the surface of the infected endothelial cell. Subsequently platelets bind to the virus glycoproteins present on the cell surface via $\alpha v\beta 3$ integrins. This integrin is proven to be the hantavirus receptor and loss of function seems to contribute to increased permeability of the endothelium in a mechanism by which vitronectin is removed from its physiological binding place on the integrin receptor. Recent work from Raftery et al. showed the release of neutrophil extracellular traps (NETs) from neutrophils upon binding of hantaviruses to integrin receptors present on neutrophils. These NETs are known to have pro coagulant effects on both primary and secondary haemostasis (65). The mechanism by which hantaviruses cause these changes in coagulation remains unknown.

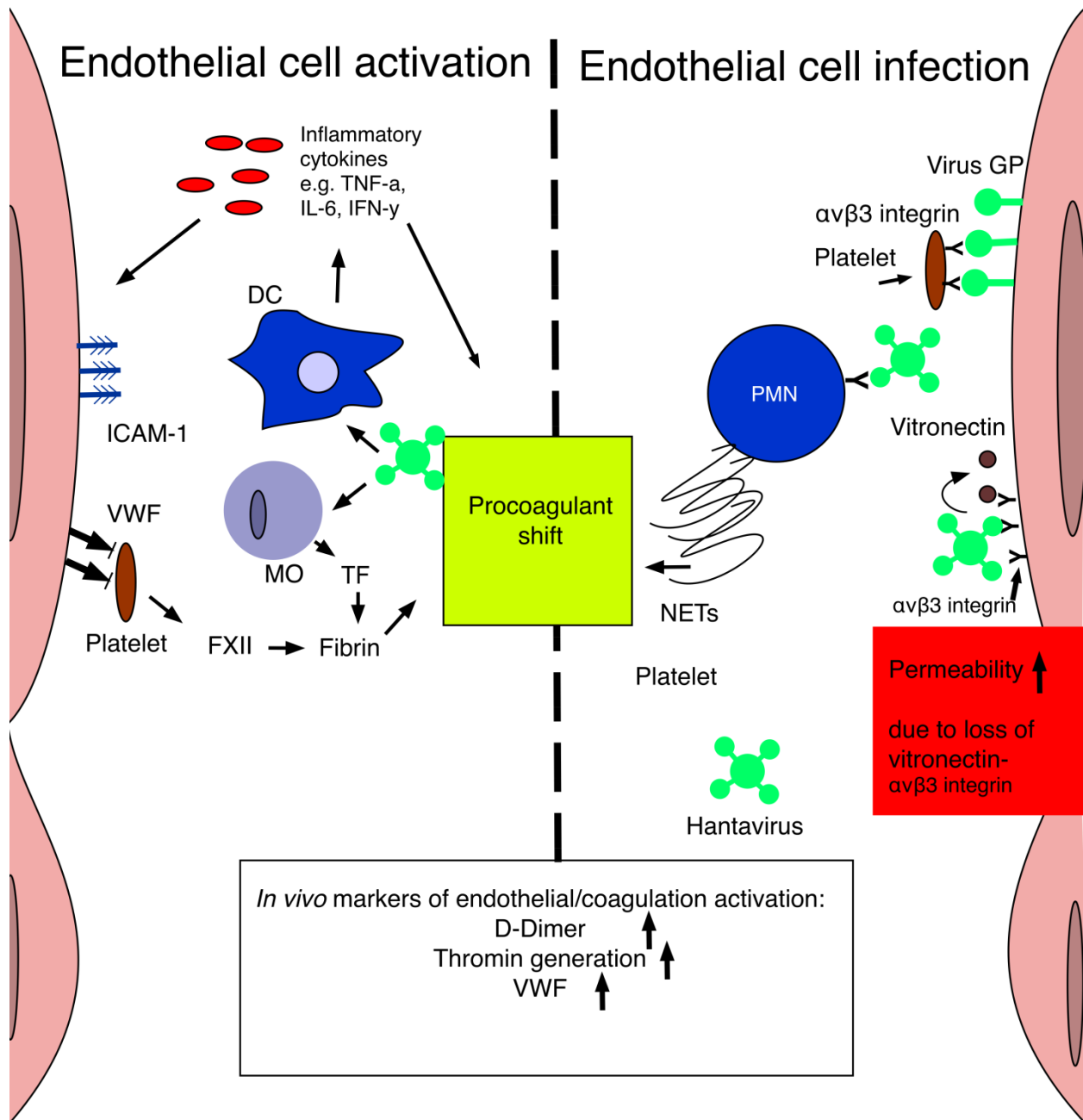


Figure 2. Summary of the current knowledge on the interaction between haemostasis, endothelial cells and hantavirus infection

The changes in haemostasis seen during hantavirus infection are the result of two different pathways. At first, dendritic cells (DC) and macrophages (MO) produce pro-inflammatory cytokines during hantavirus infection promoting the procoagulant shift. Platelets bind to hantavirus infected cells via virus glycoproteins present on the infected cells. Furthermore, polymorphonuclear neutrophils (PMN) release neutrophil extracellular traps (NETs) upon binding of hantaviruses, which on their turn continue to stimulate platelet aggregation and secondary coagulation.

Influenza virus infection

Like many respiratory infections influenza virus infections tend to cause a procoagulant shift in the haemostatic balance. Due to the magnitude of the population at risk for influenza virus infection and the solid evidence of the association between influenza virus infection and vascular thrombosis, influenza can be seen as an important representative of virus associated thrombosis. There are three manifestations of influenza in humans: seasonal, avian and pandemic influenza. Seasonal influenza is caused by influenza A or B viruses, which infect 5-15% of the human population every year (66). Symptoms vary from mild respiratory complaints up to fatal respiratory distress. Symptoms depend largely, however, on the health and immune status of the infected individual and the pathogenicity of the specific virus involved. Coagulation wise patients infected with an influenza A virus have been reported to suffer from disseminated intravascular coagulation and pulmonary micro-embolism in rare cases (67;68). In the latest outbreak of pandemic H1N1 influenza, multiple thrombotic complications were reported, such as deep venous thrombosis and pulmonary embolism. In several cases the thrombotic events resulted in bleeding complication such as pulmonary haemorrhage with haemoptysis, haematemesis, petechial rash and one case of disseminated petechial brain haemorrhage (69-78). There is a strong increased risk for the development of cardiovascular disease during or shortly after influenza virus infection, an observation that has been confirmed in large epidemiological studies and vaccination intervention trials using of seasonal influenza vaccines (79). It seems the driver behind the increased risk of thrombosis is an imbalance in haemostasis manifested by a procoagulant state (80-82). Indications for the increased clotting come from clinical, experimental mouse and *in vitro* data. Clinical reports range from mild increased coagulation and fibrinolysis markers such as VWF and D-dimer levels, to disseminated intravascular coagulation observed in severe avian influenza (83-85). Experimental mouse data indicate a procoagulant state characterized by increased thrombin generation, fibrin deposition, and an impaired fibrinolysis (86;87). How frequently influenza infection leads to clinically relevant thrombotic disease and in what magnitude and how the coagulation cascade is activated have yet to be investigated.

Aim and outline of this thesis

In this thesis the relation between viral infections and mechanisms of thrombosis and bleeding were investigated, focussing on viral haemorrhagic fever and infections known to cause thrombosis. More specifically, this thesis focussed on three essential areas of research in this field, namely: "Epidemiology, pathogenesis and prevention". Hantaviruses were chosen as a useful model for viral haemorrhagic fever pathogens while influenza was chosen as a representative virus for viral infections associated with thrombosis. The thesis starts with the

epidemiological studies related to hantavirus infection. The direct cause for us to start studying hantavirus infections was a clinical case discussed in the introduction of **Chapter 2.1**. Unique features in this case report, from presenting symptoms to diagnosis and course of disease, triggered us to further look into the epidemiology, diagnosis and treatment of these neglected rodent-borne haemorrhagic fever pathogens. This resulted to the design of the Hanta-Hunting study to test the possibility of hantavirus unawareness in the Netherlands, discussed in **Chapter 2.2**. Eventually the results from this study made us expand the study design to a cohort study in the Republic of Suriname of which the results are presented in **Chapter 2.3**.

After the section on epidemiology of this thesis we discuss pathogenesis studies pathogenesis in **Chapter 3**: “clotting and haemorrhage in infectious diseases”. We start with the *in vitro* analysis of direct effects of hantavirus infection on the haemostatic function of endothelial cells in **Chapter 3.1** followed by a helicopter view approach of the proteome of these hantavirus infected endothelial cells in **Chapter 3.2**. For this proteomics study we used the rodent-borne haemorrhagic fever pathogen *Leptospira interrogans* as a control, since it is a bacteria that causes a comparable disease while this pathogen has a very different infection and replication cyclus in humans compared to hantaviruses. Results from this non-virus control infection suggested both common and different endothelial cell effects, which we further discuss in **Chapter 3.3** by using an *in vivo – in vitro – in vivo* study design. The chapter on pathogenesis is concluded with **Chapter 3.4** where an in depth study on coagulation activation during a virus associated with thrombosis is described. For this purpose we analysed infection with different influenza viruses in the ferret model, while focussing on both circulatory and tissue haemostasis. This thesis ends with a section on how to prevent these emerging pathogens responsible for haemorrhage and/or thrombosis. In **Chapter 4.1** a large collaborative international study on the association between selenium concentration and hantavirus infections in mainland China, a country severely affected by hantavirus infections, is discussed. In **Chapter 4.2** we discussed the results of a phase 1/2a clinical trial on one of the most effective ways of prevention: vaccination making use of a modified vaccinia Ankara (MVA) platform suitable for many of the emerging and dangerous viruses studied in this thesis. Finally, **Chapter 5** is a summarizing discussion of the findings described in this thesis.

CHAPTER 2

EPIDEMIOLOGY:

“Viral Haemorrhagic Fever in the Netherlands and republic of Suriname”

Consists of:

- 2.1 A viral haemorrhagic fever case in the Netherlands and review of the literature
- 2.2 The hanta hunting study: underdiagnosis of Puumala hantavirus infections in symptomatic non-travelling leptospirosis-suspected patients in the Netherlands
- 2.3 Hantavirus and chikungunya as newly identified causes of haemorrhagic fever in the republic of Surinam.

CHAPTER 2.1

A viral haemorrhagic fever case in the Netherlands and review of the literature

Taken in part from:

‘An unusual cause of an usual presentation’

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Rodent-borne haemorrhagic fevers: under-recognised, widely spread and preventable - epidemiology, diagnostics and treatment

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CASE REPORT

Case A is a 61 year old HIV positive male who presented with fever and extreme tiredness since one week. Additional complaints were myalgia, headache and macroscopic haematuria. On physical examination the patient was ill with a temperature of 40 °C, blood pressure 135/84 mmHg, pulse rate of 82/min and had bilateral flank tenderness. The patient's medical history shows a well regulated HIV status with an undetectable viral load and a repeated deep venous thrombosis for which he used acenocoumarol. The patient had not been abroad but recently visited his vacation home in the eastern part of The Netherlands. Laboratory values are listed in Table 1. Urinalysis showed proteinuria and haematuria. On suspicion of a possible auto-immune acute renal failure, antineutrophil cytoplasmic antibodies, antinuclear-antibody and anti-glomerular basal membrane were determined acutely, which were negative. Empirical treatment with antibiotics was started after obtaining blood cultures. While fever and other physical complaints resolved after a few days, renal function did not improve initially. Therefore a kidney biopsy was performed (Figure 1) which showed a nonspecific acute inflammation with lymphocyte invasion and tubular necrosis. However renal function improved soon and the patient was discharged. Several days later he visited the outpatient clinic with high fever and malaise again. A swollen right leg was present due to a recurrent deep vein thrombosis, after anticoagulant therapy was temporarily interrupted for the kidney biopsy procedure. Based on the history with high fever and acute renal failure, leptospirosis and hantavirus infections were considered. Serology for leptospirosis was negative, but the patient tested positive for the presence of IgM antibodies against Puumala hantavirus. In conclusion the diagnosis of nephropathia epidemica was confirmed. After a total period of 6 weeks with periods of relapsing fever the patient renal function recovered completely. A second serum sample, taken six weeks later, showed a seroconversion confirmed by the presence of IgG antibodies against Puumala hantavirus.

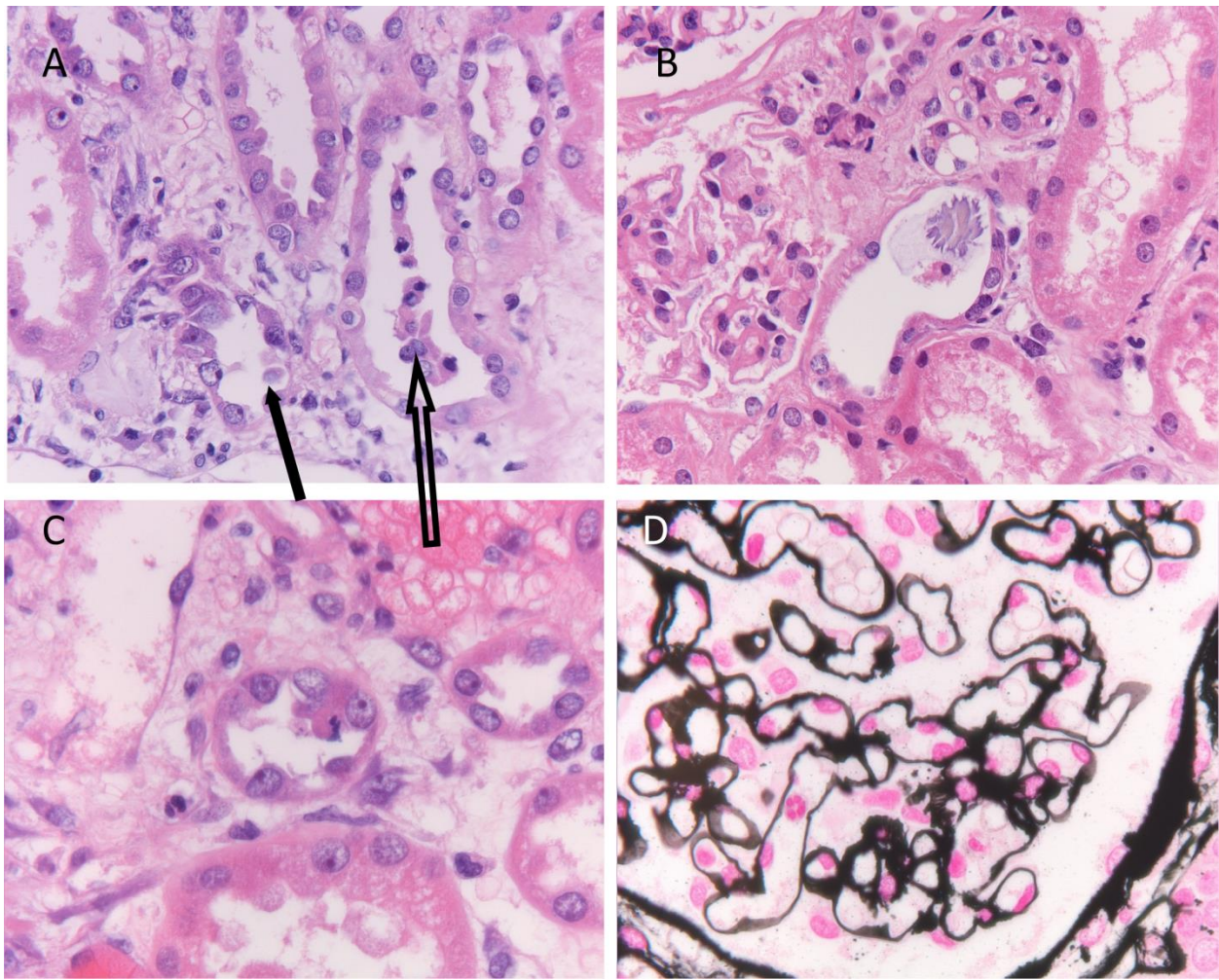


Figure 1. Renal biopsy taken at acute phase of the disease. The biopsy shows a patchy tubulointerstitial lymphocytic infiltrate, but no tubulitis A pleomorphic tubular epithelial cells with enlarged nuclei (black arrow). Sludging of necrotic epithelial cells in the tubular lumen (open arrow) B Crystalline matter in tubular lumen. C Mitosis of tubular epithelial cells, indicative of epithelial repair in acute tubular necrosis. Detail of glomerulus (400x, PAS-methenamine stain). Glomeruli were normal, showing well-expanded glomerular capillaries and normal glomerular basement membranes, without spikes or irregularities indicative of immune-complex depositions. Immune fluorescence was negative for complement (C3, C1q), light chains (kappa, lambda), and heavy chains (IgG, IgM, IgA).

Review of the literature on rodent-borne haemorrhagic fever

Due to increased international travelling and adventurous tourism to regions with conditions that are conducive for transmitting diseases, imported infectious haemorrhagic fever has become a serious challenge for clinicians and laboratory research (88;89). Viral and bacterial pathogens that cause haemorrhagic fever have the potency to result in fulminant disease, accompanied by high mortality. A significant part of these haemorrhagic fevers originate from rodents as their reservoir host. For this reason the routes of disease transmission, epidemiological patterns and preventative measures overlap significantly. Although outbreaks of rodent-borne haemorrhagic fever and imported cases have been increasingly reported during the past decade, epidemiological and clinical studies are still scarce for several diseases (90;91). The clinical picture of the rodent-borne haemorrhagic fevers, particularly in the early phase, is rather non-specific, leading to misdiagnosis and under recognition. The efficacy of treatment strategies is mainly based on small groups of patients or case reports, rather than randomised control trials. This means that making treatment protocols is more anecdotal than evidence based. Vaccines are often unavailable or will not have been approved by the authorities. This review summarises the epidemiology, diagnosis and treatment of hantaviruses, *Leptospira*, Lassa virus and New World arenaviruses as the most significant rodent-borne haemorrhagic fevers. Knowledge of (local) epidemiology, clinical symptoms and adequate diagnostics will aid the clinician in correctly recognising and diagnosing rodent-borne haemorrhagic fever in patients. Furthermore, adequate treatment and supportive care, preventative measures and new developments are discussed.

Methods

Citations were retrieved from PubMed and MEDLINE databases and from locally accessible files of the KIT Royal Tropical Institute Library Amsterdam, the Netherlands. After thorough evaluation we chose the pathogens proven to cause haemorrhagic fever and being directly transmitted from rodents to humans (not needing a vector). Databases were searched using the search terms “hantavirus”, “Leptospirosis”, “Lassa Fever”, “South-American Haemorrhagic Fever”, in combination with “clinical symptoms”, “treatment” and “epidemiology”. Titles, abstracts and references were scanned for relevance and implication for clinical settings using the following criteria: “epidemiology, clinical symptoms, diagnosis and/or treatment of rodent-borne haemorrhagic fever pathogens”.

Hantaviruses

Hantaviruses are distributed worldwide. The disease was first reported in Asia and later recognised in Europe, and the Americas, and seems to be under-recognised in Africa (54). Two known syndromes are caused by hantaviruses: haemorrhagic fever with renal syndrome (HFRS), with the milder subtype nephropathia epidemica (NE) and the hantavirus cardiopulmonary syndrome (HCPS). HFRS is endemic in large parts of Eastern and Northern Europe and Asia, while small clustered outbreaks of HCPS have been reported in North and South America (92;93). NE is prevalent in Europe and transcontinental countries like Russia (54). While we describe the generally accepted separated hantavirus syndromes recent publications give rise to the thought that typical symptoms defining HFRS and HPS can actually be caused by both Old and New-World hantaviruses (94).

Transmission and host

Hantaviruses are spread from infected rodents to humans (95). Transmission occurs through the inhalation of aerosols of virus-contaminated faeces or urine. Infection through rodent bites via infectious saliva or tissue handling in laboratory settings has also been reported, but is rare (95-97). There are at least 54 hantaviruses of which no less than 22 are known to cause disease in humans (98). While for some serotypes there is an ongoing debate about their pathogenicity in humans like the Tula virus. Each hantavirus seems to be strictly related to one host species, or to closely related host species, like the striped field mouse (*Apodemus agrarius*) for Hantaanvirus that causes HFRS in Asia, the bank vole (*Myodes glareolus*) for the Puumala virus (PUUV) that causes mild HFRS/NE in Europe and the deer mouse (*Peromyscus maniculatus*) for the New-World Sin Nombre virus, which is one of the causative agents of HCPS (99-101). With the exception of spill-over infection between the reservoirs of Dobrava-Aa and Dobrava-Af (102) and in addition to rodents, insectivores; mainly shrews and moles, are a large group of natural reservoir hosts for often non-pathogenic hantaviruses (103). Also two different hantaviruses have been isolated in Africa from bats. However transmission to humans has not yet been proven (104). Humans are a dead-end host and do not seem to shed the virus. However, human-to-human transmission has been reported in some hantavirus cases of Andes infection and Andes virus antigen has been detected in both the secretory cells of the salivary glands as in urine from patients with HCPS (105;106). While PUUV RNA has also been isolated from the saliva of HFRS patients raising the question inter-human transmission may occur (107). Andes virus seems to have a lower sensitivity to the antiviral effect of human saliva compared to other hantaviruses (108). Case reports of hantavirus infections during pregnancy did not include vertical transmission (109). The distribution and incidence of hantavirus infections in humans depends on the availability and the density of the rodent host. Incidence of PUUV caused NE is highest in Fennoscandia but in recent years outbreaks have occurred in Western and Central-Europe, in addition to the Balkan area. While in Russia, certain regions close to the

Volga River PUUV infections are highly endemic. Next to PUUV three other pathogenic hantaviruses circulate in Europe being: “Dobrava-Belgrade (with the subtypes Dobrava-Ap, Dobrava Af and the milder variant Dobrava-Aa) and Seoul”. Dobrava-Belgrade transmitted by the yellow-necked mouse (*Apodemus flavicollis*), infections are reported from South-East Europe with incidence being highest in “Croatia, Slovenia, European Russia, Bosnia-Herzegovina and Hungary” while Dobrava-Aa is more restricted to the North-Eastern parts of Europe like the Baltic States and Russia (103). Seoul virus infections in humans appear very rare in Europe apart from laboratory acquired infections. However there are reports describing detection of Seoul virus antibodies in *Rattus rattus* and *Rattus norvegicus* Belgium, Portugal and France (110;111). Hantavirus studies from Africa are limited, however there is evidence for hantavirus circulation in West-Africa (Guinea, Cote D’Ivoire). Hantaviruses detected in Africa seem to be closely related to other Murinae, Old-world mice and rats, associated hantaviruses (112). Risk factors for hantavirus infection are related to the exposure to rodents and the size of the rodent population (109;113-116). Presence of rodents or their droppings in and around a home significantly increases this risk (117). In addition using rodent traps, visiting forestry areas and participating in rural/recreational activities increase the risk of infection (118-122). Other risk factors include the handling of firewood, living close to a forest (<50 metres), staying in a house which is easy accessible for rodents or re-entering and cleaning a holiday house/cottage after the winter (119;122). For both the New and Old-world hantaviruses environmental and occupational factors play a role (119;120;122-124). Besides the risk associated with occupational exposure, particularly in the military, farming industry, and for forest workers and mammalogists/veterinarians, occasional specific outbreaks have been reported in people working with rodents in a laboratory setting (125-127). Studies in Europe have shown a correlation between smoking and hantavirus infection, possibly due to impaired ciliary activity in the respiratory tract facilitating hantavirus infection (120;122;128). The prevention of rodent infestation and rodent control seem to be the best way to reduce infection risk (125;129).

Clinical picture and diagnosis

Haemorrhagic fever with renal syndrome (HFRS):

HFRS is recognisable by the triad of fever, renal failure and haemorrhage. The disease can be divided into five phases: febrile, hypotensive, oliguric, diuretic, and convalescent. HFRS patients first develop a *flu-like* syndrome accompanied with atypical complaints like fever, myalgia, headache and flank pain. The hypotensive phase is thought to be the result of haemodynamic alterations, due to an impaired vascular tone and increased vascular permeability associated with pathological findings of pulmonary oedema and retroperitoneal edema (130). Renal failure in HFRS often develops in a late acute stage (131;132). Kidney biopsies show a damaged medulla, interstitial oedema and haemorrhage, together with

cytotoxic T-cell infiltrates and the extravasation of erythrocytes (133). An elevated, left-shifted, serum leukocyte count and elevated C-reactive protein (CRP) up to a high level such as in bacterial infection, are present. Patients with HFRS may develop serious haemorrhagic complications, starting with petechiae, epistaxis and mucosal bleedings and in a later phase gastrointestinal bleedings, haematuria and pleural bleedings. More rare haemorrhagic events are right atrial haemorrhage (57), spleen haemorrhage (58) or pituitary haemorrhage with endocrinal disturbance or even panhypopituitarism (59;60). Coagulation disorders range from mild thrombocytopenia to diffuse intravascular coagulation (DIC) (134). An in-depth Korean study among HFRS patients showed the prolongation of bleeding time, prothrombin time and partial thromboplastin time, together with decreased plasma activity of factor II, V, VIII, IX, X (61).

Depending on the hantavirus species case fatality rates in HFRS vary from 0.1 to 15% in hospitalised cases, where patients die due to circulatory and/or renal failure (101). Asymptomatic cases occur around five to ten times more frequently than symptomatic cases in HFRS (98). However, this depends on the type of hantavirus and on human factors. Dobrava-Belgrade is known to be the most pathogenic of the Old-World hantaviruses, whereas Puumala causes NE, a milder form of HFRS with CFR of 0.1%. Due to a long incubation time, typically 2-6 weeks, infections contracted in areas endemic for a particular hantavirus may obscure differential diagnosis elsewhere.

Nephropathia epidemica (NE):

Nephropathia epidemica is considered to be a mild form of HFRS caused by PUUV with a CFR below 1% and haemorrhagic manifestations in only about 10% of the symptomatic patients. Up to 80% of the infections with PUUV remain asymptomatic. Patients who do develop the disease usually present with fever and acute renal failure. Accompanying symptoms are abdominal pain, nausea, blurred vision, flank pain and headache. Most patients with NE recover from the acute renal failure within 6 months. Follow-up studies have proven an increased hypertension risk >10 years post infection (135-139). Respiratory complaints can also arise during NE (140). A lung biopsy will show a local immune response with elevated expression of VCAM-1 (141). Clinical chemistry may show high serum creatinine and urea, haematuria and proteinuria in the acute phase. More than half of the patients presenting with NE show cardiac involvement. This varies from abnormalities on the electrocardiogram (t-top inversion) to, in rare cases, pericardial effusion (142;143). In severe cases NE patients may develop encephalitis (144) or more haemorrhages like petechiae, haematuria or epistaxis (145). Patients infected with PUUV showed coagulation activation, marked by an almost 24-fold D-dimer increase (134). Disease severity was reported to correlate with plasma IL-6 levels instead of CRP (146). Procalcitonin is also elevated in PUUV infections. The broad range in serum procalcitonin in PUUV makes it unsuitable to distinguish from bacterial infection. However, there is a correlation between

procalcitonin and the severity NE (147). The symptoms in children do not differ greatly from adults with HFRS/NE (148). However, NE does seem to take a less severe course in children (149).

Hanta cardiopulmonary syndrome (HCPS):

HCPS-causing hantaviruses mainly target the lungs (150;151). HCPS begins with signs of fever, myalgia and respiratory symptoms, followed by the acute onset of severe respiratory distress (152;153). Clinical characteristics are not specific and are therefore not useful when differentiating between HCPS and other diseases. However, the combination of dizziness, nausea and the absence of a cough with a low platelet count, low serum bicarbonate and elevated haematocrit can be typical of a HCPS case (154). Besides respiratory distress, patients with HCPS show a decreased cardiac output and dysrhythmias (151). HCPS is characterised by oedema due to endothelial dysfunction instead of severe haemorrhage. Patients could develop thrombocytopenia and bleeding complications, but respiratory distress due to oedema is the main cause of death. CT images may show thickening of interlobular septa, ground-glass opacities, and occasionally small ill-defined nodular opacities (152;153). The acute onset of the respiratory distress leads to hypoxia, cardiac insufficiency, high intubation rates and a need for inotropics (117;155). Case-fatality rates vary from 30-60% in hospitalised cases (117;155;156). However, recent studies have shown that extracorporeal membrane oxygenation (ECMO) seems to drastically decrease mortality rates in HCPS (157). Histopathological examination of the lungs shows interstitial and alveolar oedema, sporadic alveolar haemorrhage, and mild interstitial pneumonia (158). The clinical picture in children does not seem to differ from that in adults with hantavirus cardiopulmonary syndrome (159).

Hantavirus disease

Recent papers debate the absolute difference between HFRS and HCPS hantavirus syndromes. It seemed in many cases that symptoms overlap and HFRS cases presented with acute respiratory failure without signs of kidney involvement while HCPS patients may show renal complications (160). Therefore it is suggested to use the term “hantavirus disease” for all hantavirus related described syndromes.

Diagnostics

Since almost all acute cases have IgM and IgG antibodies against the nucleocapsid protein of hantaviruses, serological diagnostics are most commonly used for verifying hantavirus infection using indirect IgG and IgM enzyme-linked immunosorbent assays (ELISA), IgM capture ELISAs or immunofluorescence assays (IFA) (54;151;161). A high level of cross reactivity exists in hantavirus serology. Cross reactivity occurs within the group of Serotype 1 “Dobrava-Belgrade virus, Saaremaa virus, Seoul virus and Hantaan virus” and within the Serotype 2 group

“Puumala virus, Tula virus, Topografov virus and Sin-Nombre-like viruses” (162). Because of this high level of cross reactivity, a focus reduction neutralisation test (FRNT) remains the gold standard in hantavirus serology. However, neutralising antibodies are not yet produced in the acute phase of infection. More recently, a replication reduction neutralisation test (RRNT), which is based on a quantitative RT-PCR technique, showed promising results in efficiently and rapidly detecting hantavirus-neutralising antibodies (163). PCR can be performed on blood and serum samples during the first days after the onset of the symptoms. One-step RT-PCR assays for hantavirus detection showed high sensitivity and specificity (98;164). However, the molecular diagnosis of acute infections can be difficult, since the viraemic stage is often limited to the incubation period prior to the onset of the symptoms and shortly afterwards (165). Rapid immunochromatographic IgM-antibody diagnostic tests have been evaluated in different studies. Hujakka et al. found a 96-100% sensitivity and specificity for the specific immunoassays developed for Dobrava, Puumala and Hantaan hantaviruses. Combined rapid tests are less sensitive (166).

Treatment

In HFRS the initiation of prompt and proper supportive treatment is crucial. This support comprises measures such as monitoring fluid balance, diuresis, kidney function and the use of FFP/transfusions in the treatment of haemorrhagic complications (167;168). Ribavirin treatment can be useful in the very early phase of HFRS by reducing the risk of haemorrhagic events and the severity of renal insufficiency (169-171). Treatment with interferon only inhibits the virus in-vitro, while adjunctive prednisolone treatment showed no beneficial outcome (172;173). An open-label trial and a double-blind clinical trial only showed the adverse effects of the ribavirin treatment in HCPS, but no beneficial effects (174);(175). For the time being, the treatment of HCPS will mainly depend on critical care management which includes the avoidance of fluid overload, vasopressors to maintain cardiac output, and the use of extracorporeal membrane oxygenation in severe cases (98;175;176). Trials in Asia have shown a promising reduction of HFRS, with conventional and molecular vaccines, but these have not been approved by the responsible authorities (177;178). Problems for developing a European vaccine arise from the different circulating serovars (177;178). No vaccine for HCPS has been tested in humans so far, but it is believed that individuals surviving a hantavirus infection develop lifelong immunity (177;178).

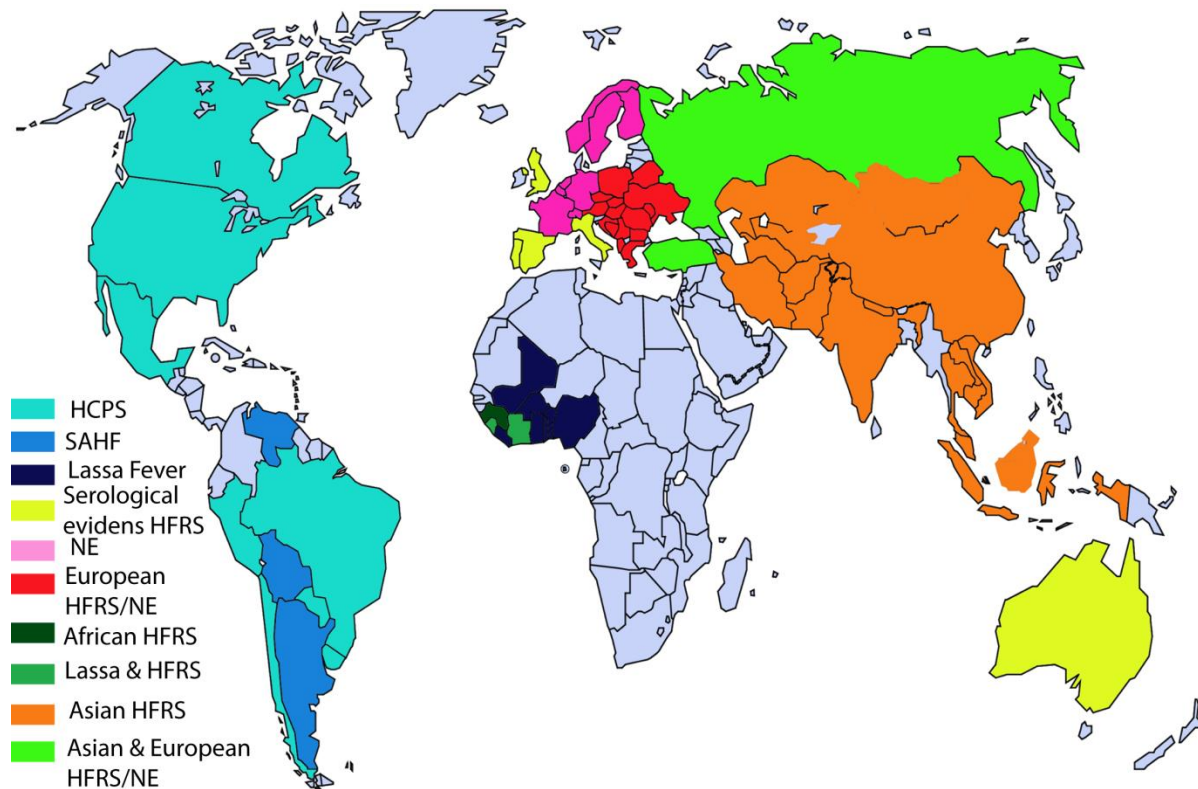


Figure 3. Global distribution of the rodent-borne haemorrhagic fever pathogens. Knowledge of the global distribution is important for clinicians. For instance travelers returning from South America may be infected with the SAHF viruses, HCPS viruses and *Leptospira*, whereas Lassa fever or NE would be considered highly unlikely. Leptospirosis, not added in this figure, occurs on every continent, with higher incidence in (sub)tropical areas.

Leptospirosis

Leptospirosis has been recognised as an emerging infectious disease with severe repercussions all over the world. Large outbreaks are closely related to heavy rainfall and flooding. The global incidence of endemic leptospirosis has been estimated at 5 per 100,000 head of population and that of epidemic leptospirosis at 14 per 100,000 (Report of the Second Meeting of the Leptospirosis Burden Epidemiology Reference Group, World Health organization, 2011, Geneva). Incidence rates are the highest in tropical areas such as southern Asia and South America (179;180). The disease has also emerged as an illness that impacts adventure tourists, especially those who participate in water sports. Incidence rates may vary due to the difficult diagnosis of leptospirosis and unfamiliarity, and the fact that incidence rates can vary significantly per year (88;179).

Transmission and host

Pathogenic *Leptospira* enter the environment through the shedding of bacteria via the urine of chronically infected hosts (181;182). Two different transmission routes are described: a) indirectly, through contact with infected water or soil, which is probably the main route for most serovars, and b) directly, through contact with infected animal urine or tissues (183;184). Different hosts can carry *Leptospira* serovars (185;186). Rodents and insectivores, particularly rats, are thought to be the major carrier-animals, but dogs, pigs, cattle and horses are also considered to be reservoirs. Infected rodents stay asymptomatic and are thought to chronically shed *Leptospira* (182). Infection through abraded skin in humans seems to be the most common portal of entry (187-189). However, infection can also occur after contact with exposed mucous membranes of the nose, mouth and eyes (189;190). The presence of *Leptospira* in mesenteric lymph nodes in autopsy studies is suggestive for an oral transmission route for leptospirosis (188). Infections with *Leptospira* are reported from all over the world. The infection risk most frequently correlates with rainfall, flooding, soil saturation and stagnant water pools on walking paths (182;191-193). Walking barefoot or living close to a sewer or trash belt also increases the risk of infection. Moreover, the incidence in many regions is at its highest during the rainy season. Outbreaks are seen after natural disasters like flooding and hurricanes (194). Rural and jungle areas in particular seem to provide the environmental conditions that are the most conducive for the survival and transmission of leptospires. However, urban leptospirosis occurs in areas with high rodent densities like city slums (195;196). The incidence is the highest in resource limited settings in Asia, South-America and probably Africa however data is limited. In Europe the highest incidence rates are reported from European part of Russia, Croatia and Ukraine (Pappas et al. 2008). Studies show a higher infection incidence in men, and this is the highest in men of working age (20-60 years old). This observation is probably due to occupational risk factors (197;198). During the past decades, recreational exposure is a factor of growing importance (199). Recreational activities related to leptospirosis infection include water-related activities such as canoeing, rafting and swimming (200-203). Indeed, outbreaks after sports events, mainly sports which includes water contact like triathlon or rafting, have been reported (188;200;204). There are reports of adventure tourists who were infected after jungle tracking or cave exploring, or a specific occurrence of wading through small streams in a mangrove forest in Malaysia(205;206).

Clinical picture and diagnosis

The presentation of patients with acute leptospirosis can vary greatly (182;207). Infections often remain asymptomatic or patients will develop a mild disease. Acute leptospirosis usually presents with one or more of the following symptoms: a fever of sudden onset, chills, headache, severe myalgia, conjunctival suffusion, anorexia, nausea, vomiting, and prostration (179). Severe disease is rapidly progressive with a high case-fatality rate. The classic triad of

icterus, kidney failure and haemorrhagic diathesis is often recognised as Weil's disease (208). However, atypical presentation of severe disease constitutes an important pitfall. The icterus seen in leptospirosis seems to be the result of a septic cholestasis instead of acute hepatitis. Indeed, liver function tests are only mildly impaired during acute disease. Severe leptospirosis can cause life threatening bleeding complications. There is evidence that both an impaired primary- and secondary haemostasis play roles. Primary haemostasis is impaired, due to thrombocytopenia, which frequently occurs during leptospirosis infection (209). Furthermore, patients with severe leptospirosis often have a prolonged activated partial thromboplastin time (APTT) and raised thrombotic markers like D-Dimer and Prothrombin up to DIC, according to the International Society on Thrombosis & Haemostasis criteria (207). Despite these disturbances in primary and secondary haemostasis there seems to be a role for endothelial cell dysfunction in leptospirosis. *In vitro* pathogenic *Leptospira* interfere with the endothelial cell barrier function (210). A severe form of bleeding disorders in leptospirosis is the Leptospirosis Associated Pulmonary Haemorrhage Syndrome (LPHS). This syndrome is a major cause of haemorrhagic fever in developing countries, causing a fulminant presentation with heavy pulmonary bleeding and mortality ranging up to 75 or 80% (211;212). Symptoms are coughing, haemoptysis, often recognised at a late stage after intubation, and in around 50% of the cases there is a patchy infiltration seen on X-thorax. Based on clinical symptoms, leptospirosis is hard to distinguish from other endemic pathogens. During several outbreaks leptospirosis has often been under-recognised and misdiagnosed with, for example, dengue fever. CRP is elevated in acute phase of severe leptospirosis. Furthermore, there are some promising studies about experimental markers (soluble ST-2, long pentraxin-3 (PTX3) and procalcitonin) (213-215).

Diagnostics

The gold-standard test is the Microscopic Agglutination Test (MAT). This test requires a dark-field microscope to determine agglutination titres (216-218). Because leptospirosis antibodies are formed 5 to 7 days after the onset of the disease, MAT is particularly accurate in its later phases (216;217). Paired serum testing is necessary to determine seroconversion or a significant rise in titre. MAT titres can be indicative for infecting serogroups. However, the MAT is laborious, expensive and needs highly trained personal, so it can only be performed in specialised laboratories. The results of an MAT may be compromised by background titres in patients from endemic regions and cross-agglutinating antibodies (212;217;219). A combination of MAT with Enzyme-linked immunosorbent assay (ELISA) for the detection of IgM antibodies against *Leptospira* increases sensitivity (218;220). PCR is useful in the first 5 days of the disease and a validated RT-PCR is available (216). Cultures are usually not used for clinical practice but they do have relevance for the identification of reservoir hosts and epidemiological studies and they can be retrieved from blood and CSF fluids before the tenth day of the illness. Usually urine cultures can be positive after the seventh day of the disease (218;221). There are multiple

rapid tests for leptospirosis diagnostics. For example, antibody-coated gold nanoparticles for the early detection of *Leptospira* in urine has proven highly sensitive and relatively cheap way of early diagnosis compared to PCR (217). Latex-based agglutination rapid tests show a sensitivity of around 70% in the early phase of the disease, becoming even more sensitive later (222). Immunochromatography-based rapid tests based on crude antigen, if properly used, can show good sensitivity (223). However, in endemic regions, due to high seroprevalence, such a format can have a limited value (224).

Treatment

Treatment with penicillin G results in lower morbidity and causes no harmful side effects in acute leptospirosis. The treatment is most effective during the first five days after symptom development (225). However, most cases still develop severe disease either due to late start of treatment or treatment failure. Doxycycline has been proven to be an alternative to penicillin, although it is prescribed for mild cases (226). Prophylactic doxycycline reduces severe complications of leptospirosis and symptomatic disease. However, there is a debate as to whether it prevents infection or only affects the severity of the disease (88;227). The side effects of a weekly 200mg oral dosage of doxycycline should be taken into account (227;228). The treatment of icteric leptospirosis and LPHS is more complicated and should be restricted to tertiary centres, if available (229). In these severe forms of leptospirosis supportive care is often necessary (229;230). Besides mechanical ventilation, inotropic therapy and renal dialysis, treatment of the bleeding disorders can be considered with fresh frozen plasma (FFP) and thrombocyte transfusions.

Lassa Fever

Lassa fever affects two to three million people in West Africa annually, with an estimated CFR of 15-20% in hospitalised cases. It was first described in 1969 in Lassa, a town in northeast Nigeria, as causative agent of a haemorrhagic fever causing fulminant disease. Although only 20-30 imported cases to Europe are described, most of them were fatal, mainly due to lack of awareness of the risk, delay in diagnosis and delay in initiation of therapy (231). While nosocomial transmission often occurs in West Africa, imported cases of Lassa in-to Europe have never shown human-to-human transmission in hospital settings, with the exception of seroconversion in a healthcare worker in 2003 (232). Lassa fever is endemic in Guinea, Sierra Leone, Nigeria, Liberia and Ivory Coast. The disease also occurs in Ghana, Senegal, Togo, Benin, Mali and Burkina Faso.(233-236) The disease is caused by the Old-World arenavirus Lassa; an enveloped, single-stranded, bi-segmented RNA virus (237). The virus has a small and a large genome segment. Four lineages are identified: Josiah (Sierra Leone), GA391 (Nigeria), LP (Nigeria) and strain AV, which was isolated when imported into Germany and is closely related to Josiah (231;238).

Transmission and host

Lassa virus is restricted to one host, the African multimammate rat (*Mastomys natalensis*), other *Mastomys* species do not seem to shed the virus (239). This rodent breeds frequently, produces large numbers of offspring and is distributed widely throughout west, central, and the eastern parts of the African continent (240). *Mastomys natalensis* is a commensal rodent, readily colonising human settlements, thereby increasing the risk of rodent-human contact. When infected, these rodents carry and excrete the virus lifelong. Human infection occurs after inhalation of aerosolised excreta (often urine), consuming contaminated foods or by direct contact with abraded skin (237;241). Human-to-human transmission occurs through direct contact with blood or bodily secretions from infected persons. Living close to someone who had signs of infection in the past 12 months, was associated with almost a twofold infection risk in Guinea (242). Working in a hospital or laboratory without proper hygiene precautions also increases the risk of infection (243;244). These findings confirm the need for barrier nursing techniques and strict hygiene measures (245). Other risk factors include activities that increase rodent contact, such as poor housing conditions and rodent infestation (246). An exacerbating factor is that the multimammate rat is a known delicacy and food source in West Africa. Hunting them and consuming their meat is therefore a considerable risk factor (247).

Clinical picture and diagnosis

About 80% of patients infected with the Lassa fever virus will develop subclinical or mild disease. After the incubation period of 1 to 3 weeks patients first develop a high fever, general weakness and malaise. These symptoms occur typically 4-7 days upon the onset of disease

followed by a typical pharyngitis (with yellow-white patches) and more non-specific symptoms such as headache, back-, chest- or abdominal pain, nausea and vomiting, diarrhoea, conjunctivitis, coughing and proteinuria (248-250). In endemic areas, the best discriminating symptoms in this early phase seem to be the combination of proteinuria and pharyngitis (160;161). When progressing into the next stage (after 7 days), clinical hallmarks typically include a facial oedema, neurological disorders, such as convulsions and encephalopathy and bleeding. Haemorrhagic manifestations vary from mucosal bleedings (gums, nose eyes) to severe internal bleeding from the stomach, bowel, kidney, brain and heart. These conditions only occur in one third of the patients and are associated with death (244;251). There are no data supporting evidence for a DIC in severe Lassa, since coagulation markers are almost always within normal range. Haemorrhage seems to be the result of endothelial cell dysfunction induced by a direct or indirect toxic effect on the endothelial cell by the virus and by inflammatory proteins (cytokines). Indeed, post-mortem findings show a generally damaged vascular endothelium, characterised by oedema in the submucosa, subcapsular haemorrhages in the liver, haemorrhage and oedema in the myocardium, gross oedema in the intestine and a focal pneumonitis (248);(252). There is some evidence that a diminished primary haemostasis may add to the haemorrhagic diathesis since acute-phase plasma from Lassa fever patients show the inhibition of APD, collagen and sodium-arachidonate-induced platelet aggregation (241). Moreover, plasma derived from patients with Lassa fever inhibits aggregation and thromboxane generation in normal platelets (253). In this stage with severe haemorrhages, shock and sometimes encephalitis, CFR increases to 38-52% (254;255). Patients may die in the late stage (>14 days) from shock and respiratory distress due to pleural effusion, possibly complicated with haemoptysis. When patients recover, deafness, hair loss and long-term psychiatric complications are reported (251;254).

Diagnostics

The first choice of diagnostics is an ELISA detecting anti-Lassa IgM and/or IgG antibodies (256). ELISA has shown sensitivity up to a 100% in experimental settings (256;257). Due to a persistent high viraemia for at least 10-15 days, PCR techniques are useful during a prolonged time in Lassa infection. Viral RNA has been isolated from patients' semen up to three months post infection (258). However, due to a high sequence diversity (up to 30%) between Lassa virus strains false negative results cannot be excluded (257).

Treatment

Ribavirin is considered effective in Lassa fever (251;259). The treatment is effective in any stage of the disease (259;260). A ten-day dosage of 15 mg per kg bodyweight in acute disease was shown to effectively decrease mortality, CFR and morbidity (261). The treatment should start as soon as possible, preferably in the first six days of the disease. Intravenous therapy has a better

clinical outcome than oral ribavirin (251;261). Ribavirin was also proven useful as a high-risk post-exposure prophylaxis (PEP). A dose of 800 mg, once a day, proved to be as effective as PEP in a small cohort (n=23) from Liberia (262).

Despite the availability of antiviral treatment additional supportive care is often needed. Fluid replacement and blood transfusions, in particular, may be necessary (263;264). No vaccination is available yet but there are promising results with a replication-competent vaccine expressing the Lassa virus glycoproteins (265).

South-American haemorrhagic fevers (SAHF)

Isolated in 1958, the Junin virus (JUNV) was held responsible for a severe haemorrhagic syndrome that caused dengue-like symptoms in a clustered outbreak in Argentina. Argentinean haemorrhagic fever (AHF) is endemic in a limited area in the Pampas in Argentina. Outbreaks occur from March to June. Its incidence sharply decreased after the availability of an effective vaccine and neutralising antibody treatment (266;267). JUNV was the first of the New World arenavirus to be discovered. Other SAHF viruses include: Machupo, the causative agent of Bolivian haemorrhagic fever (BHF), Guanarito, the causative agent of Venezuelan haemorrhagic fever (VHF), and Sabia virus, the causative agent of a haemorrhagic fever case in Brazil. These New World arenaviruses are, in general, confined to certain areas and rodent-host species. VHF, whose causative agent Guanarito virus was isolated in 1989, occurs in a rather well defined area in central Venezuela and peaks every year during harvest season (from November to January) (268). The causative agent of BHF, the Machupo virus, was isolated in 1964 after an outbreak with severe haemorrhagic disease with a CFR around 20%. This disease can also have outbreaks in rural areas, often related to agricultural work taking place during the dry season (from May to October).

Transmission and host

Transmission is thought to occur in a similar way to hantavirus and Lassa disease, i.e. through infective aerosols (266;267). Person-to-person transmission and nosocomial infections have been described for BHF (91;267). The main natural reservoirs of Junin virus (AHF) in Argentina are the dry land vesper mouse (*Calomys musculinus*) and the small vesper mouse (*Calomys laucha*). For the Machupo virus this is the large vesper mouse (*Calomys callosus*) and for Guanarito virus the short tailed zygodont (*Zygodontomys brevicauda*). The Sabia virus has only been related to one human case of the disease and the natural reservoir is, as yet, unknown (267;269). Risk factors are mainly linked to agricultural work and rodent-infested villages. Adult male agricultural workers are mostly at risk, but children and other adults of both sexes can also contract the disease. Despite several laboratory infections there are no reports of SAHF cases outside of the SAHF endemic areas. As the incubation periods for SAHF vary from 7-14 days, travel-related import to non-endemic countries might occur (90).

Clinical picture and diagnosis

After an incubation period of 7-14 days patients with AHF, BHF and/or VHF first develop a non-specific flu-like syndrome. Other symptoms include coughing, prostration, abdominal pain, lumbar and muscle pain, severe headache, conjunctival injection, facial flushing, and generalised lymphadenopathy (268;270;271). Compared to Lassa, patients with SAHF have a higher tendency to develop serious neurological signs, such as encephalitis and cranial haemorrhages. Haemorrhagic complications consist mainly of petechiae, conjunctival haemorrhages, mucosal bleeding and gastrointestinal bleeding with melena. Bleeding normally starts after five days of illness. Laboratory results show thrombocytopenia, leukocytopenia and alterations in coagulation markers (e.g. prolonged partial thromboplastin time) (272). The increased bleeding tendency is thought to be related to both impaired primary haemostasis (thrombocytopenia and impaired platelet function) and secondary haemostasis (267;273). In AHF a prothrombotic phenotype is seen with elevated procoagulant markers (D-dimer), thrombin-antithrombin complexes (TAT) and thrombin fragment 1 and 2 (F1+F2). A raise in serum PAI-1 indicates inhibition of fibrinolysis in severe cases (274). The role of vascular endothelium dysfunction in SAHF has been studied *in vitro*. The up-regulation of ICAM-1 and VCAM-1 expression on Junin-virus-infected endothelial cells, the increased production of nitric oxide and prostaglandin PGI₂ and the reduced production of coagulation factors (e.g. von Willebrand factor) are shown *in vitro* (273).

Early diagnosis in SAHF is possible by RT-PCR as patients show viraemia for 5-10 days after the first symptoms. Molecular detection has proven to be sensitive in the early phase of AHF (a sensitivity of 98% and a specificity of 76%) (275). The gold-standard test is a proven seroconversion by ELISA or IFA using paired sera. Antibodies against the highly conserved nucleocapsid proteins (NP) of New World arenaviruses are commonly used for virus detection because the SAHF viruses occur very locally. However, this cross reactivity could be a limitation in diagnosing a returning traveller who visited multiple SAHF endemic areas.

Treatment

During the first eight days of the disease AHF can be treated with immune plasma containing high titres of neutralising antibodies (276). Also, Ribavirin can be effective in AHF as in the other SAHF (277-279). Further treatment should consist of supportive care regarding blood loss in haemorrhagic events and adequate handling of neurological complications such as seizures. An effective live-attenuated vaccine is available for AHF (280).

Co-Infections

The rodent-borne haemorrhagic fevers share several characteristics including areas of endemicity, transmission pathways and the majority of their clinical symptoms. Therefore, the possibility of co-infection with multiple rodent-borne pathogens can't be excluded in differential diagnostics. However, reports on such co-infections are very rare. Two Reports from Croatia describe double infections with both *Leptospira* and a hantavirus in humans (281;282). Interestingly both pathogens have been isolated from the same reservoir rodent, namely *Apodemus flavicollis* (Dobrava-Belgrade and Leptospira) and from a *Myodes glareolus* in Croatia (Puumala and Leptospira) (283;284). Co-infections between other rodent-borne haemorrhagic fever pathogens have not been reported. There are also reports describing co- and multi-infections with *Leptospira* and other pathogens such as dengue virus, malaria parasites, hepatitis A and E virus, *Orientia tsutsugamushi*, *Toxoplasma gondii*, *Babesia* and *Francisella tularensis* in humans and/or non-human reservoir species (284-291). The possibility of multiple pathogens in one patient can be both of clinical and diagnostic importance. Case reports showed more severe disease in co-infected patients and sometimes the occurrence of a co-infection warrants changes in treatment (290;292). Co-infections between Hanta and Lassa fevers have not been reported. However outbreaks of these viral diseases have poorly been investigated for the occurrence of co-infections. While in the Guinean Forests of West-Africa, an ecological very diverse region reaching from Guinea and Sierra Leone eastward to Cameroon, serological evidence is present both the Lassa virus infections as infections with the Sangassou hantavirus (SANV) (293).

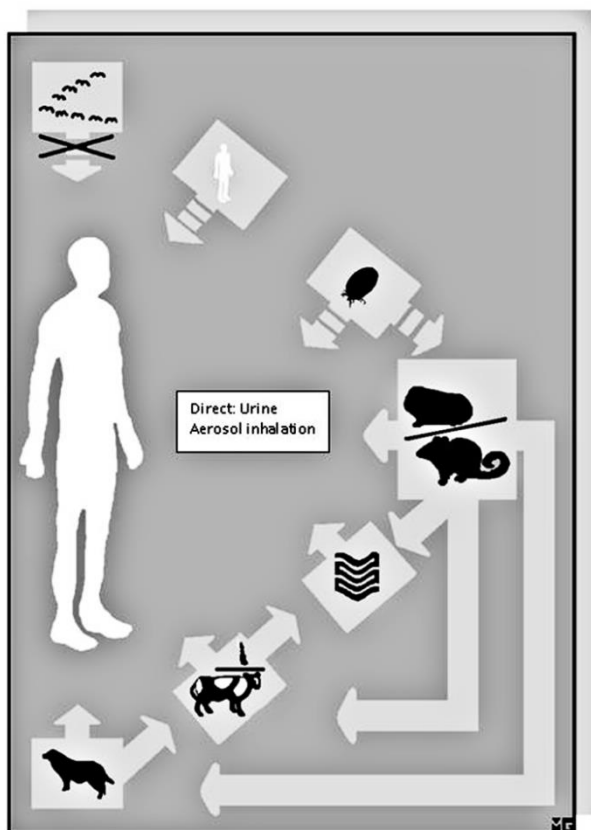


Figure 4. Routes of transmission in rodent-borne haemorrhagic fever. *Transmission of Rodent Borne haemorrhagic fever causing pathogens can occur directly via aerosol inhalation (hanta, Lassa, SAHF) or contact with skin and mucosa (leptospirosis). Furthermore indirect transmission is possible by non-rodent animals (often in leptospirosis). Birds do not seem to play a role in transmission routes of leptospirosis. Hantaviruses and the new and old-world arenaviruses were thought to be restricted to rodent reservoir hosts. However, hantaviruses have also recently been isolated from insectivores and African bats, although transmission to human has not been proven yet.*

Concluding remarks

The rodent-borne haemorrhagic-fever pathogens belong to an important group of zoonotic diseases causing severe disease all over the world. Since the diseases described in this review share many common clinical symptoms and laboratory markers, and to some extent show an overlap in their geographic distribution, accurate diagnosis depends on the availability of sensitive and specific testing and high-level clinical practice. Rodent control, proper education aimed at avoiding contact with rodents or rodent excreta and the development of vaccines can decrease infection rates, such as in AHF. However, for these preventive measures to be effective, thorough knowledge of the pathogens, and particularly their specific rodent hosts, is necessary. Changes in climate and human behaviour (particularly land use) are predictors for the distribution of cases or outbreaks of rodent-borne pathogens outside their current areas of endemicity (294). The challenge lies in the timely recognition of the disease, followed by adequate risk management. Knowledge of the clinical picture, epidemiology (including rodent ecology) and pathogenesis of these diseases are vital if we are to decrease morbidity and mortality (295). Proper knowledge of their epidemiology facilitates the choice of adequate diagnostics for the right pathogen.

CHAPTER 2.2

The Hanta-Hunting study: underdiagnosis of Puumala hantavirus infections in symptomatic non-travelling leptospirosis-suspected patients in the Netherlands, in 2010 and April to November 2011

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Abstract

“Leptospirosis and haemorrhagic fever with renal syndrome (HFRS) are hard to distinguish clinically since these two important rodent-borne zoonoses share hallmark symptoms such as renal failure and haemorrhage. Leptospirosis is caused by infection with a spirochete while HFRS is the result of an infection with certain hantaviruses. Both diseases are relatively rare in the Netherlands. Increased incidence of HFRS has been observed since 2007 in countries that border the Netherlands. Since a similar rise in incidence has not been registered in the Netherlands, we hypothesise that due to overlapping clinical manifestations, hantavirus infections may be confused with leptospirosis, leading to underdiagnosis. Therefore, we tested a cohort of non-travelling Dutch patients with symptoms compatible with leptospirosis, but with a negative diagnosis, during 2010 and from April to November 2011. Sera were screened with pan-hantavirus IgG and IgM enzyme-linked immunosorbent assays (ELISAs). Sera with IgM reactivity were tested by immunofluorescence assay (IFA). ELISA (IgM positive) and IFA results were confirmed using focus reduction neutralisation tests (FRNTs). We found hantavirus-specific IgG and/or IgM antibodies in 4.3% (11/255) of samples taken in 2010 and in 4.1% (6/146) of the samples during the 2011 period. After FRNT confirmation, seven patients were classed as having acute Puumala virus infections. A review of hantavirus diagnostic requests revealed that at least three of the seven confirmed acute cases as well as seven probable acute cases of hantavirus infection were missed in the Netherlands during the study period”.

Introduction

Hantaviruses, negative-stranded RNA viruses belonging to the *Bunyaviridae* family, can cause severe disease in humans. Depending on the type of hantavirus, either haemorrhagic fever with renal syndrome (HFRS) or hantavirus cardiopulmonary syndrome may occur after inhalation of virus-containing aerosols (296). HFRS is characterised by acute renal failure, fever (above 38.5 °C) and potentially accompanied by severe bleeding complications (98); it is a notifiable disease in the Netherlands. HFRS cases are found in large parts of Europe and Asia (297). Pathogenic hantaviruses are rodent-borne and each of these viruses are spread by a specific rodent species. For the HFRS-causing hantaviruses, these include *Apodemus*, *Myodes*, *Rattus* and possibly *Microtus* species (298). The causative agent of HFRS known to be endemic in the Netherlands is Puumala virus (PUUV), which is spread by its chronically infected reservoir, the bank vole (*Myodes glareolus*) (299). Symptomatic cases of PUUV infection may develop mild HFRS, often referred to as nephropathia epidemica. Recent reports describe PUUV infections with a broader clinical spectrum, ranging from mild febrile cases, without renal impairment or haemorrhage, to severe respiratory manifestations without any signs of renal involvement (300;301). Historically, the occurrence of PUUV infection in the Netherlands has been restricted to the eastern and southern parts of the country, with an incidence of 6–30 cases reported per year (approximately 0.04–0.18 cases per 100,000 population) (302). Since 2007, several studies have described an increase in the number of human PUUV infections in neighbouring countries at the eastern (Germany) and southern borders (Belgium) (303;304). To date, a similar increase in the number of human PUUV infections has not been observed in the Netherlands.

In the early 1990s, Groen et al. tested 8,892 sera obtained in the Netherlands from different risk groups, such as renal disease patients, and subjects from suspected occupational risk groups, such as forestry workers and military personnel, for the presence of hantavirus antibodies (305). The highest prevalence (up to 6%) was seen in participants with known occupational risk factors associated with increased rodent exposure (299;305). Data (which are as yet unpublished but a summary of the main results is available) from a large serum bank study in the Netherlands that started in 2006 showed a hantavirus seroprevalence, in a cross-sectional population based study, of 1.7% (306). Given that 70–80% of PUUV infections are asymptomatic and that only 5–10% of symptomatic patients will probably seek medical attention, the 25–30 cases reported every year in the Netherlands (with a population of 16.8 million) are indicative of potential underdiagnosis of hantavirus infections (299;307). In 2011, we described a case report of a patient with nephropathia epidemica diagnosed outside the area known to be endemic for hantavirus circulation in the Netherlands, Overijssel (308). Although the patient had visited a known PUUV-endemic area, this information in the patient history did not result in rapid diagnosis of the cause of the disease, illustrating unawareness of hantavirus infections.

Leptospira, a genus of helical-shaped bacteria, forms another important group of causative agents of rodent-borne haemorrhagic fever in the Netherlands (309). Pathogenic *Leptospira* cause leptospirosis, which shares many clinical manifestations with HFRS, such as renal failure, thrombocytopenia and potential bleeding complications (297). Interestingly, two studies, from Italy and Sri Lanka, showed an increased hantavirus seroprevalence in patients suspected of having leptospirosis. Compared with control groups consisting of office personnel or healthy blood donors, the number of confirmed cases of hantavirus infection was significantly higher among those who were clinically suspected, by a clinician, of having leptospirosis (310;311). Groen et al. reported a hantavirus seroprevalence of about 1% in patients suspected of having acute leptospirosis in the Netherlands in samples collected between 1972 and 1994. The actual prevalence of confirmed acute leptospirosis was slightly higher (3%) (305).

In the Netherlands, a relatively low number of leptospirosis cases are registered annually, as are HFRS cases, with a reported incidence of 0.25 leptospirosis cases/100,000 population (309). To investigate the putative underdiagnosis of hantavirus infection in symptomatic patients, we tested a cohort of leptospirosis-suspected, but confirmed-negative, patients with no travel history for the previous three months, for the presence of hantavirus-specific antibodies, using a two-step strategy: pan-hantavirus enzyme-linked immunosorbent assays (ELISAs) followed by two separate immunofluorescence assays (IFA): one to detect PUUV serogroup antibodies and one to detect Hantaan virus (HNTV) serogroup antibodies (HNTV was used as it belongs to the same serogroup as Seoul hantavirus (SEOV). Recent evidence indicates the circulation of SEOV in Europe (312-314), spread by *Rattus norvegicus*, which is also a well-known carrier of *Leptospira* (297). To confirm the ELISA and IFA results, focus reduction neutralisation tests (FRNTs) were used, the gold standard technique in hantavirus serology. All IFA IgM-positive sera were tested in the FRNT with PUUV virus. In addition, because recent evidence indicates the circulation of SEOV in Europe, we included SEOV, as well as Dobrava virus (DOBV), in the FRNT, although not the main aim of this study, to gain insight into the potential introduction of these viruses in the Netherlands.

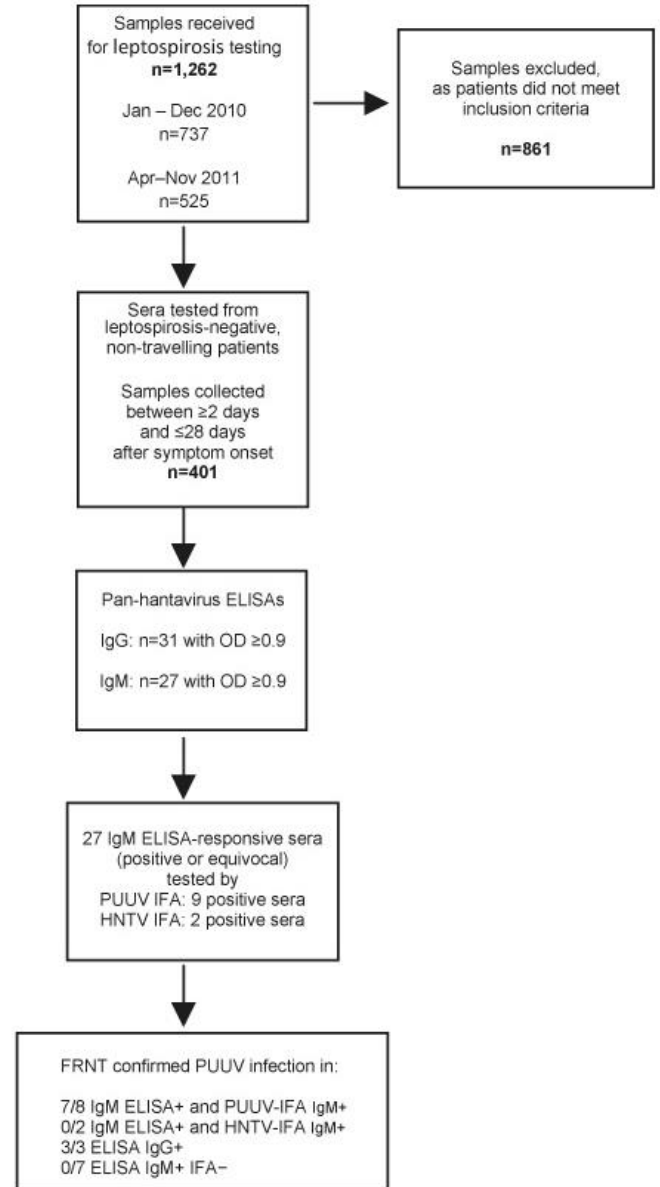
Methods

Serum bank

Sera from non-travelling Dutch patients with a negative leptospirosis diagnosis – based on a microscopic agglutination test (MAT), ELISA and culture performed at the National Leptospirosis Reference laboratory (NRL) at the Royal Tropical Institute (KIT) in Amsterdam – were included in our study. A sample was deemed to be negative for leptospirosis if the patient did not meet the case definition for leptospirosis – i.e. the in-house ELISA for leptospirosis was below the cut-off titre of 1:80 and the MAT showed no relevant titre of *Leptospira*-specific antibodies (<1:160) (315). The study cohort was taken from submissions of sera to the NRL in 2010, as a large increase in the number of PUUV infections in Germany and Belgium were observed that year (298). In addition, we also included sera from patients meeting the above inclusion criteria that were received by the NRL during April to November 2011, the season for PUUV activity in northern and western Europe (316) (Figure 1).

Figure 1. Design, inclusion criteria and confirmatory steps of the hanta hunting study, the Netherlands, 2010 and April–November 2011

As we were interested in patients who had pan-hantavirus IgM antibodies, we selected patients whose samples had been collected at least two days after symptom onset, up to four weeks (28 days) after symptom onset. All samples were heat inactivated (30 minutes at 56 °C) and stored at –20 °C until testing. Requests for leptospirosis testing were accompanied by a standardised form with information about place of residence, travel history, presenting symptoms and occupation: these data were reviewed.



Hantavirus underdiagnosis was assessed by checking if testing for hantavirus was requested at either of the hantavirus diagnostic laboratories in the Netherlands (Erasmus MC in Rotterdam and RIVM in Bilthoven) for any of the pan-hantavirus ELISA-responsive sera (equivocal or positive result in an IgG or IgM ELISA).

Patients whose sera were responsive in any of the diagnostic tests were ranked by likelihood of hantavirus infection. Patients whose sera were positive in the IgM ELISA, IFA and FRNT were considered a confirmed case of acute hantavirus infection. If only the IgM ELISA was positive (or equivocal) and IFA was positive, the patient was considered a probable acute case. If only the IgG ELISA was positive and therefore IFA was not performed, but the FRNT was positive for PUUV, the patient was also considered a probable acute case. If only the IgM ELISA was positive or equivocal, the patient was considered not a case of hantavirus infection.

Enzyme-linked immunosorbent assay

Sera were screened using pan-hantavirus IgG and IgM DxSelect ELISAs (Focus Diagnostics). These ELISAs are used for testing a broad range of hantaviruses, although there are variations in sensitivity and specificity per specific hantavirus. According to the material supplied by the manufacturer, the IgM test has an overall sensitivity of 95.1% (83.5–99.4%) and a specificity of 94.1% (83.8–98.8%). The IgG test has comparable performance characteristics, with an overall specificity of 95% (91.4–100%) and a sensitivity of 95% (75–98%); both tests are compared with a reference ELISA by external investigators. For the Netherlands, the performance of these ELISAs in detecting antibodies to PUUV and, potentially, SEOV, is of importance. Data supplied by the manufacturer showed a sensitivity of 70% (45.7–88.1%) in the IgM ELISA and 95% (83.2–100%) in the IgG ELISA for PUUV-specific antibodies, as tested by FRNT. For SEOV FRNT-positive samples, the sensitivity was 50% (11.8–88.2%) in the IgM ELISA and 95% (54.1–100%) in the IgG ELISA. An optical density (OD) of > 1.10 was regarded as positive, between ≥ 0.90 and ≤ 1.10 as equivocal and <0.90 as negative.

Immunofluorescence assay

ELISA IgM-positive or equivocal sera were tested in IFA by using commercial slides with PUUV- and HNTV-infected cells (PROGEN Biotechnik). Only IgM-reactive samples were chosen as these are indicative of a recent infection, possibly related to the clinical symptoms that were the basis of the initial request for leptospirosis testing. IFA was used because of its higher reported specificity and the possibility of being able to distinguish between PUUV- or HTNV-like serotype infections (manufacturer's insert, PROGEN Biotechnik). Before testing, the sera were incubated with liver acetone powder from calves (Sigma-Aldrich, Germany) to reduce background fluorescence. For the IgM test, the sera were pretreated with GullSORB (Meridian Bioscience Inc., United States) to reduce isotype competition. Sera were serially diluted twofold starting at

1:32 and incubated on the slides for 1 hour at 37 °C. After this step, the wells were incubated with either a fluorescein isothiocyanate (FITC)-labelled goat anti-human IgG or IgM conjugate. Fluorescence was scored under an immunofluorescence microscope. The cut-off titre for a positive result was defined as the sample dilution for which specific fluorescence was greater than the sample dilution for which specific fluorescence was just identifiable: in this study, it was >1:64.

Focus reduction neutralisation test

All samples positive in the pan-hantavirus IgM ELISA and PUUV IgM IFA were selected for FRNT confirmation (samples 4, 7, 10, 11, 12, 14, 19, 21 and 22). We also selected two samples positive or equivocal in the pan-hantavirus IgM ELISA and positive in the IgM HNTV IFA for FRNT (samples 5 and 6). In addition, eight samples that were positive in the IgM ELISA but negative in the IFAs were also selected for FRNT (samples 2, 3, 9, 13, 15, 17, 18 and 20). As a fourth category, three samples that had not been included in the IFA analysis (as they were ELISA IgM negative), but that tested positive in the IgG ELISA (samples 1,8 and 16), were selected for FRNT confirmation. FRNTs for DOBV strain Slovenia, SEOV strain 80-39 and PUUV strain Kazaan were carried out as described elsewhere (317). Diluted sera were mixed with an equal volume of diluted virus containing 30–70 focus-forming units/100 µl. The serum end-concentration was 1:40. The mixture was incubated at 37 °C for 1 hour and subsequently inoculated into wells of six-well tissue culture plates containing confluent Vero E6 cell monolayers. The wells were overlaid with a mixture of agarose and tissue culture medium and incubated for 7–13 days. The agarose was removed from the wells and the cells were fixed. For PUUV-infected cells, polyclonal macaque serum (318) was used as the primary antibody and the monoclonal antibody 1C12 for DOBV- and SEOV-infected cells as described elsewhere (319). This step was followed by adding peroxidase-labelled goat-anti-human IgG for the macaque serum and goat-anti-mouse IgG for the 1C12 monoclonal antibody to the cells, to indicate virus-infected cells. Tetramethylbenzidine was used as substrate and foci were counted. An 80% reduction in the number of foci, compared with the virus control, was used as the criterion for virus neutralisation titres.

Review of hantavirus diagnostic requests carried out during suspicion of leptospirosis at the time of sampling

All samples responsive in the IgM and/or IgG pan-hantavirus ELISA were checked for patient-specific characteristics (sex and date of birth). The combination of sex and date of birth was checked in the databases of the hantavirus diagnostic laboratories in the Netherlands. If the sample combination matched the information from the database and the diagnostic request was made in 2010 or 2011, the patient was scored as having been adequately diagnosed for hantavirus disease during the onset of their symptoms. If the sex and date of birth combination

could not be found in the databases, but the patient's sample was reactive in any of our tests, the case was scored as a missed probable or confirmed hantavirus case, as described in the serum bank section above.

Ethical issues

This study was exempted from ethical review of human subject research by the Medical Ethical Review Committee of the Erasmus MC Medical Centre, University of Rotterdam. All data have been anonymised and are not attributable to individual patients.

Results

Enzyme-linked immunosorbent assay and immunofluorescence assay serology

Of the 1,262 samples received for leptospirosis diagnostic testing during January– December 2010 and April–November 2011, 861 were excluded, as the patients did not meet the inclusion criteria of our study.

All selected and available sera (n=401) were tested by pan-hantavirus ELISAs. Overall, the IgG ELISA resulted in 18 positive and 13 equivocal samples. The IgM testing resulted in 17 positive and 10 equivocal samples. A total of 11 samples reacted in both the IgG and IgM ELISAs, bringing the total number of samples that responded in both ELISAs to 47, i.e. 11.7% of the 401 samples (4.3% (11/255) of samples taken in 2010 and 4.1% (6/146) of the samples taken in 2011).

Subsequently, the 27 samples with a positive or equivocal response in the IgM ELISA were tested using both PUUV and HTNV IFAs. In total, nine of the 27 IgM ELISA-responsive samples tested positive for both PUUV IgM and IgG by IFA.

Interestingly, two serum samples were positive in the HTNV IFA, but negative in the PUUV IFA, despite repeated PUUV testing. One of the HTNV-positive samples tested positive for both HNTV IgG and IgM with titres of 1:512; the other sample was positive only for HNTV IgM, with a titre of 1:128.

Confirmation by focus reduction neutralisation test

FRNT was performed on eight of the nine sera with a positive IgM response in the PUUV IFA (there was an insufficient amount of serum in the ninth sample). It confirmed that seven of the eight samples tested were from patients with recent PUUV infections (samples 4, 7, 10, 11, 12, 19 and 22) (Table 1).

Table 1. Serological test results from samples selected for focus reduction neutralisation test confirmation of hantavirus infection, the Netherlands, 2010 and April–November 2011 (n=22)

Sample number	Date of sampling	ELISA ^a		IFA ^b				FRNT ^c			Acute hantavirus infection case status
		IgM	IgG	PUUV IgM	PUUV IgG	HNTV IgM	HNTV IgG	PUUV	SEOV	DOBV	
PUUV IFA IgM positive											
4	May 2010	POS	POS	1:128	1:128	NEG	1:128	POS	NEG	NEG	Confirmed
7	Jul 2010	POS	POS	1:128	1:128	1:128	NEG	POS	NEG	NEG	Confirmed
10	Aug 2010	POS	POS	1:128	1:128	NEG	1:128	POS	NEG	NEG	Confirmed
11	Aug 2010	POS	POS	1:128	1:128	NEG	NEG	POS	NEG	NEG	Confirmed.
12	Aug 2010	POS	Equi	1:128	1:128	NEG	NEG	POS	NEG	NEG	Confirmed.
14	Aug 2010	POS	NEG	1:128	1:128	NEG	NEG	NEG	NEG	NEG	Probable
19	Jul 2011	POS	POS	1:128	1:128	1:128	1:128	POS	NEG	NEG	Confirmed
21	Sep 2011	POS	NEG	1:128	1:128	NEG	NEG	NS	NS	NS	Probable
22	Sep 2011	POS	Equi	1:128	1:128	1:128	1:128	POS	NEG	NEG	Confirmed
HNTV IFA IgM positive and ELISA IgM reactive											
6	Jun 2010	POS	POS	NEG	NEG	1:512	1:512	NEG	NEG	NEG	Probable
5	Jun 2010	Equi	NEG	NEG	NEG	1:128	NEG	NEG	NEG	NEG	Probable
ELISA IgM reactive and PUUV or HNTV IFA negative											
2	Feb 2010	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Not a case
3	Feb 2010	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Not a case
13	Aug 2010	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Not a case
15	Oct 2010	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Not a case
17	May 2011	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Not a case
18	Jul 2011	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Not a case
20	Aug 2011	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Not a case
9	Jul 2010	Equi	POS	NEG	NEG	NEG	NEG	NS	NS	NS	Not a case
Not tested by IFA (as ELISA IgM negative), but ELISA IgG positive											
1	Feb 2010	NEG	POS	NT	NT	NT	NT	POS	NEG	NEG	Probable ^d
8	Jul 2010	NEG	POS	NT	NT	NT	NT	POS	NEG	NEG	Probable ^d
16	Apr 2011	NEG	POS	NT	NT	NT	NT	POS	NEG	NEG	Probable ^d

We also tested the two sera with an HNTV IgM-positive IFA and a positive or equivocal ELISA IgM response (samples 5 and 6): both sera were negative by FRNT.

To test if cases had been missed due to lack of sensitivity of the IFAs, we selected eight samples, of which seven samples had enough serum left for FRNT, with only IgM reactivity in the ELISAs and a negative PUUV or HNTV IFA (samples 2, 3, 13, 15, 17, 18 and 20): all seven samples were negative by FRNT.

We also selected three samples that had not been included in the IFA analysis (as they were ELISA IgM negative), but that tested positive in the IgG ELISA (Cases 1,8 and 16). These samples were tested by FRNT because there was a high degree of suspicion of PUUV infection based on the application form for leptospirosis diagnostic request sent by the clinician (e.g. recorded renal failure and possible rodent exposure). All three patients had a long duration of their complaints (more than three weeks since symptom onset, making it possible that hantavirus disease, without the detection of IgM antibodies, was the cause of their symptoms. All three were positive in the PUUV FRNT.

Thus in total, FRNT for PUUV, SEOV and DOBV was performed on 20 of the 22 selected samples, due to an insufficient amount of serum in two samples. Of the 20 serum samples tested, 10 were confirmed as PUUV positive, seven of which were considered due to a recent infection, based on the presence of IgM antibodies.

Patient characteristics and registered clinical signs and symptoms in confirmed cases

Because of the retrospective nature of our study, we could confirm if patients had been adequately tested for hantavirus infection during their disease course or if the patient was a missed case of PUUV infection. Of the 27 samples with at least an equivocal response in the IgM ELISA in our study, which would necessitate further testing of a follow-up serum sample, four were adequately tested by routine serology for hantavirus infection at diagnostic centres at the time of sampling during suspicion of leptospirosis (samples 4, 10, 11 and 12).

The two samples that were responsive in the ELISAs and HNTV IFA, but not in FRNT (samples 5 and 6), were not tested for hantavirus antibodies by ELISA or IFA at the time the patients were sampled.

All available information from retroactively determined probable or confirmed cases of hantavirus infection that were not tested for hantavirus infection during suspicion of leptospirosis at the time of sampling is shown in Table 2. In general, most of the cases were in the eastern parts of the Netherlands. Newly recognised areas with confirmed cases were in the northern province of Groningen and the western province of Zuid-Holland. The missed confirmed and probable cases of hantavirus infection are shown in Figure 2 according to the location of sampling, ranked by likelihood of hantavirus infection, with the highest level of evidence being that of a sample with a positive response in the IgM ELISA confirmed by IFA (IgM and IgG) tests and a positive FRNT result.

Sample number	Date of request for leptospirosis testing	Sex	Age group in years	State	Information at time of request for leptospirosis testing	Retroactive hantavirus diagnostic test results			Acute hantavirus infection case status
						ELISA ^a	IFA ^b	FRNT ^c	
16	Apr 2011	M	20–24	Overijssel	Acute kidney failure and hepatitis	IgG +	NT	PUUV +	Probable
1	Feb 2010	M	20–24	Overijssel	Prolonged severe disease	IgG +	NT	PUUV +	Probable
8	Jul 2010	M	10–14	Groningen	Contact with soil water and potential rodent exposure	IgM Equi IgG +	PUUV – HNTV –	PUUV +	Probable
21	Sep 2011	F	25–30	Overijssel	Icteric; non-responsive to antibiotics	IgM +	PUUV IgM +	NS	Probable
14	Aug 2010	F	20–24	Zuid-Holland	No additional information	IgM +	PUUV IgM +	All –	Probable
5	Jun 2010	M	20–24	Limburg	No additional information	IgM +	HNTV IgM +	All –	Probable
6	Jun 2010	M	25–30	Gelderland	Emergency hospital admission, to an intensive-care unit	IgM + IgG +	HNTV IgM + HNTV IgG +	All –	Probable
22	Sep 2011	M	45–49	Gelderland	Clinical picture not understood	IgM + IgG +	PUUV IgM + PUUV IgG +	PUUV +	Confirmed
19	Jul 2011	M	50–54	Groningen	Extreme tiredness, fever and diarrhoea	IgM + IgG +	PUUV IgM + PUUV IgG +	PUUV +	Confirmed
7	Jul 2010	M	35–39	Noord-Brabant	Severe disease with high fever and emergency hospital admission	IgM + IgG +	PUUV IgM + PUUV IgG +	PUUV +	Confirmed

Table 2. Information on cases of hantavirus infection undiagnosed at time of sampling during suspicion of leptospirosis, the Netherlands, 2010 and April–November 2011

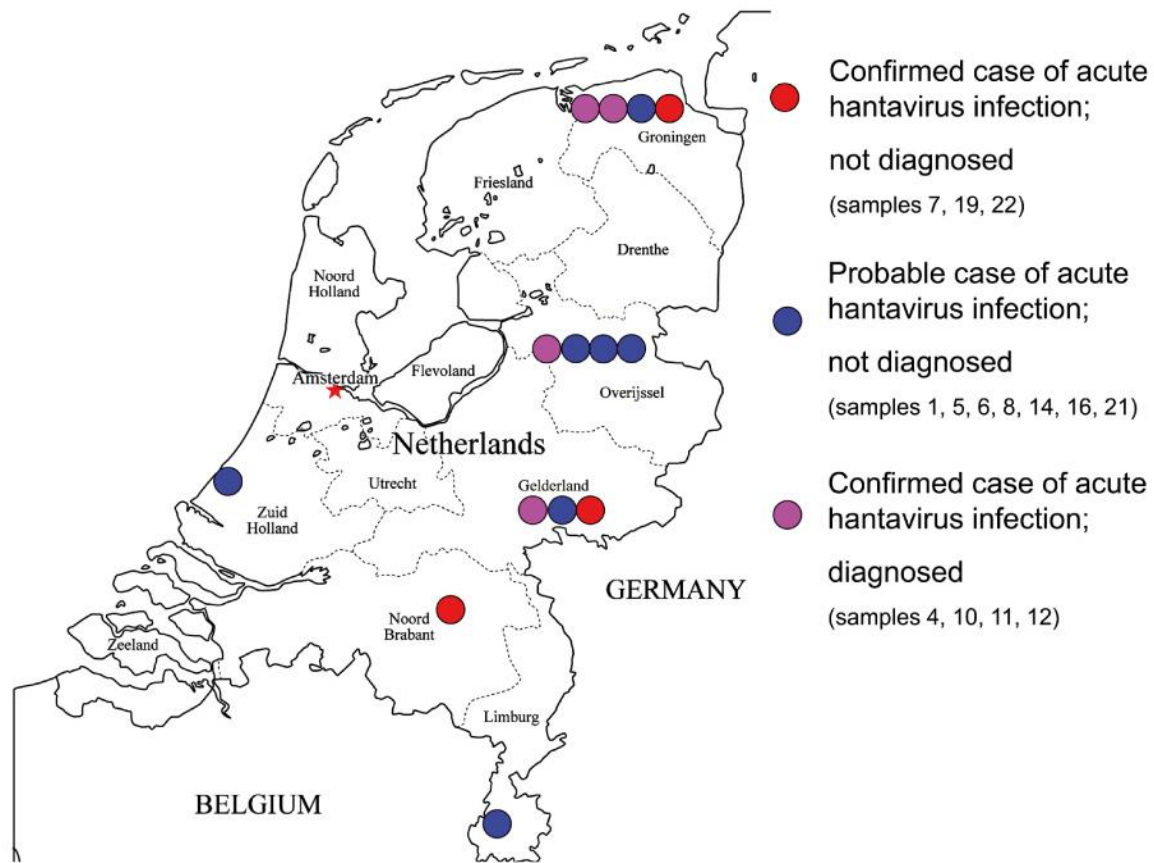


Figure 2. Distribution of probable and confirmed cases of hantavirus infection in the Netherlands, 2010 and April–November 2011 (n=14)

This figure shows the probable and confirmed cases of acute hantavirus infection in the Netherlands and if they were adequately diagnosed during their illness or if these were ‘missed’ cases. The cases are ranked by likelihood of hantavirus infection. A confirmed case of acute hantavirus infection being that of a positive IgG and IgM response in both pan-hantavirus enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) and a positive result in the focus reduction neutralisation test (FRNT). A probable case is strongly suggestive of an acute case of hantavirus based on ELISA, IFA or FRNT results, but either the FRNT was negative or we were unable to show the presence of IgM antibodies.

Discussion

In the samples tested, a positive response of hantavirus IgM antibodies in the ELISA was observed in 4.3% (11/255) of samples taken in 2010 and 4.1% (6/146) of the samples taken in 2011. When including samples with an equivocal ELISA result, the overall percentage with an IgM response was 6.7% (27/401). Confirmation with IFA IgM resulted in a 2.7% (11/401) seropositivity in the cohort. Of these 11 samples, seven were confirmed by FRNT, corresponding to an overall seropositivity in the cohort of almost 2%. However, this percentage could very well be an underestimation. For instance, one sample could not be confirmed by FRNT due to a lack of available serum after ELISA and IFA screening. It is conceivable that in our cohort of symptomatic patients, of the seven confirmed hantavirus diagnoses, at least three cases were not adequately diagnosed at the time of disease (Cases 7, 19 and 22). The other four confirmed cases were found in the databases of the hantavirus diagnostic centres in the Netherlands, and thus were adequately diagnosed at the time of disease. We also identified seven probable cases of acute hantavirus infection: we consider that hantavirus infection was a highly plausible explanation for their symptoms, but either FRNT confirmation was not performed due to the lack of serum or the presence of IgM antibodies (confirming acute infection) could not be proved by ELISA and IFA.

All samples confirmed by FRNT (n=10) only showed PUUV-neutralising activity. The vector of this hantavirus is *Myodes glareolus* (bank vole), a small, reddish rodent that inhabits large parts of the Netherlands, solely in grasslands and forests. Case 6, with high OD values in IgM and IgG ELISA screening and a positive HNTV IFA, did not neutralise PUUV, DOBV or SEOV.

This study revealed a high seroprevalence of about 2% of hantavirus antibodies in a cohort of leptospirosis-suspected patients who tested negative for leptospirosis. Leptospirosis in the Netherlands may be either endemic or imported (309). In our cohort, travel history was well documented and hence we consider it quite certain that the patients we studied contracted hantaviruses in the Netherlands.

This cohort also gave us the opportunity to study the circulation of hantaviruses other than PUUV in The Netherlands. It is important to monitor this, since evidence is mounting of an increase in the number of SEOV infections in Europe and worldwide, with a recent case reported in the United Kingdom (320). However, in the samples tested from the Netherlands, the SEOV FRNT was negative. Results were also not indicative for infections with DOBV, which is vectored by the yellow-necked mouse (*Apodemus flavicollis*). It is possible that the ELISA and IFA results in samples 5 and 6 were false positives. The specificity of the ELISA and IFA is below 100%, resulting in a (small) chance of cross-reactivity. Test specifications of the ELISA test showed no known cross-reactive pathogens, but this presumption is based on results from very small serum cohorts (manufacturer's insert). Although SEOV and DOBV have been excluded as the causative agents in our study, the remaining hantaviruses in the HNTV serogroup are

vectored by reservoir species not known to be present in the Netherlands. Thus, while we cannot rule out the possibility that other hantaviruses from the HNTV serogroup caused the disease in patients from whom samples 5 and 6 were obtained, with no travel history, this remains highly unlikely.

Our results show quite a large discrepancy between the initial ELISA screening, followed by IFA analysis and eventual gold standard FRNT confirmation. Samples that tested positive only in the IgM ELISA (n=7) were not confirmed positive by FRNT. In most cases (7/9), a positive response in the IgM pan-hantavirus ELISA in combination with a positive result in the PUUV IgM IFA was later confirmed by FRNT (sample 14 tested negative in the FRNT and sample 21 could not be tested). Therefore, we underline the importance of FRNT validation in epidemiological studies before drawing any major conclusions, particularly since hantavirus serology is highly prone to giving false-positive results (321).

Acute leptospirosis and HFRS share many clinical manifestations and certain epidemiological features. Exposure to rodents is a known risk factor for both diseases. Hallmark symptoms in both HFRS and leptospirosis include kidney failure. Two of the four cases who were diagnosed at the time of their disease course had documented kidney disorders (data not shown). However, of the 10 cases who were not diagnosed, only one (sample 16) had documented kidney failure. Of the other nine undiagnosed cases, one was described as having 'high fever' (sample 7), two as having 'severe disease' (samples 1 and 7) and one as 'clinical picture not understood' (sample 22): the fact that these cases were undiagnosed in the Netherlands during their disease course could be due to a potential lack of typical presenting symptoms for hantavirus disease in the Netherlands, meaning the absence of kidney failure. Hepatic involvement, often present in leptospirosis – one of the classic triads in Weil's disease (297) – could lead a clinician to think of leptospirosis, while not considering PUUV infection: this would have applied to the patient with hepatitis (sample 16), a probable case of acute hantavirus infection in our study. Atypical presentation of HFRS, as seen in some of the cases listed in Table 2 (samples 21 and 19), has been the subject of several recent case reports (322;323). However, unawareness and/or lack of clinicians' knowledge of how to recognise hantavirus disease could also be a reason for underdiagnosis.

Cases may also be underdiagnosed if the patients are outside the hantavirus-endemic area in the Netherlands. Such cases might not be identified due to the low, but clinically important number of infections, resulting in lack of awareness of the clinicians in these areas. If we compare the distribution of the previously undiagnosed cases in our cohort with the earlier serological data, for instance, data published by Groen et al. (305), 7 of 10 cases of hantavirus infection not tested at the time of sampling for hantavirus disease (listed in Table 2) were from outside the known endemic area. Our conclusions regarding underdiagnosis are supported by a recent study showing 1.7% hantavirus seroprevalence in the Dutch population, which should lead to more symptomatic cases than the 25–30 cases reported annually (306).

In this relatively small cohort with specific clinical indications for leptospirosis diagnostics, we have shown the presence of undiagnosed hantavirus cases. Leptospirosis itself is potentially an often-missed diagnosis in the Netherlands, due to unawareness (309). It is conceivable that physicians, who do not include leptospirosis in their differential diagnosis, are even less aware of the possibility of hantavirus infections. Vice versa, it cannot be excluded that clinicians who are aware of hantavirus infections might miss potential leptospirosis. This hypothesis could be validated by performing larger-scale serological studies with broader cohorts, comprising patients who are suspected of having leptospirosis or hantavirus infection.

On the basis of the results in this paper, we feel it is important to increase awareness of hantavirus infection in the Netherlands. The increased incidence of hantavirus infections in Europe in recent years makes this even more important. This increase is affected by a multitude of factors. Some, such as changes in landscape architecture (e.g. (de)forestation, fragmentation of land by motorways, railways and agriculture and available burrow space) and increased food availability for the rodent reservoirs, are beneficial for the spread of hantaviruses (324). The introduction or discovery of new hantavirus strains in Europe has been documented (320) and presents another major concern, necessitating epidemiological monitoring of vectors and patients. We advise that hantavirus and leptospirosis diagnostics should be considered for every patient with an undifferentiated fever in any area with potential rodent-borne infections, including typing of the causative agents if the results are positive.

By decreasing the unnecessary use of antibiotics (325) and providing clinicians with an accurate prediction of the disease course and a choice of adequate biomarkers of disease severity (324), the identification of hantavirus infections might have a limited, but important, clinical importance. Furthermore, adequately diagnosing and typing hantavirus infections is of major public health importance in order to correctly identify and educate risk groups and to design tailor-made prevention programmes, such as rodent-control programmes and changes to landscape architecture.

CHAPTER 2.3

Emerging viruses in the Republic of Suriname: retrospective and prospective study into chikungunya and hantavirus circulation, 2008-2012 and 2014.

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Submitted

Abstract

Introduction: Suriname is a country on the north-eastern Atlantic coast of South America. It is unique in the sense that different ethnic cultures live together within the country, resulting in high levels of transport of both humans and products between the Asian, African and the European continent as well as the Caribbean. Travel is only one of the many factors present in Suriname contributing to the risk for the emergence or introduction of any infectious disease. Recently circulation of both chikungunya (CHIKV) and hantavirus was reported in areas neighbouring Suriname. Here we report a retrospective and prospective study into chikungunya and hantavirus circulation. **Methods:** A chikungunya and hantavirus retrospective serological study was conducted on samples submitted for dengue, leptospirosis and/or influenza virus diagnostics between 2008 and 2012 to the Bureau of Public Health in Suriname. This was followed by a prospective CHIKV serological and molecular surveillance study until the detection of the first autochthonous CHIKV cases in Suriname in May and June 2014. **Results:** None of the tested samples showed the presence of CHIKV antibodies in the retrospective serological study. Prospective testing of CHIKV suspected patients resulted in the detection of the first autochthonous CHIKV cases in Suriname in May 2015. In one sample we were able to isolate and sequence the virus. Retrospective testing for the presence of hantavirus antibodies showed a relative high response in both pan-hantavirus ELISA and IFA. However, neutralization tests did not yield any evidence for infection with either Seoul- or Andes hantavirus. **Conclusion:** Here we report the presence of CHIKV in the republic of Suriname and the first serological indication of hantavirus infections in symptomatic patients.

Introduction

The republic of Suriname is the smallest sovereign state in South America with a population of approximately 570.000. A large variety of ethnicities are present in Suriname mainly due to its colonial history (326). Currently, the five major ethnic groups are descendants of African migration due to the slave trade (Creole and Maroon 17.7% and 14.7%, respectively), Hindustani and Javanese (27.4% and 14.6%, respectively; both descendants of labour immigrants from India (former British Indies) and Indonesia (former Dutch Indies), and mixed ethnicity (12.5%). Other nationalities present in Suriname are Chinese, indigenous Amerindians and Caucasians (326). There is a high volume of travel between Suriname and Europe (especially the Netherlands) and between Suriname and the Caribbean, which potentially contributes to the importation of any infectious disease agent. Furthermore, the relative high level of biodiversity in general in South-America and the presence of many vectors for transmission of pathogens (*Aedes* mosquitos, rodents and sandflies) make Suriname prone to vector-borne and zoonotic diseases (327;328). The Bureau of Public Health in Suriname (B.O.G.) has put much effort in the control of two endemic mosquito-borne diseases ,yellow fever and malaria, resulting in a strong reduction and almost no reported cases of these diseases in the domestic areas in the past decennium (annual report B.O.G.). Despite sporadic cases of malaria in the amazons (tropical rainforest in the inlands of Suriname) both diseases are now successfully controlled, resulting in hardly any human cases reported the past ten years. However, sporadic cases of Mayaro virus have been reported in literature. In addition, dengue is endemic in Suriname, while currently an epidemic of chikungunya virus (CHIKV) is ongoing in the Caribbean and America's. The first local transmission of CHIKV in the New World, in this case the Caribbean, was reported from the Saint Martin Island in December 2013 (329). As of January 30, 2015 a total of 1,135,892 cases have been reported in the America's of which 24,320 have been confirmed by RT-PCR, IgM IFA or a four-fold increase in CHIKV specific IgG antibodies (330). Next to fever, patients with CHIKV often show (poly)arthralgia, usually symmetric and bilateral. Arthralgia can be very severe and patients could have a long recovery resulting in a serious burden of disease.

Besides mosquito-borne diseases Suriname knows rodent-borne diseases like leptospirosis. Furthermore, the circulation of hantaviruses in adjoining countries might be indicative for circulation of hantaviruses in Surinam as well (331). Hantaviruses are often neglected, emerging viruses. Both unawareness and difficulties in both the clinical and laboratory diagnosis contribute to misdiagnosis of hantavirus cases (338). Pathogenic hantaviruses are able to cause two types of disease in humans, haemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in North and South America (297). In South America many New World hantaviruses are able to cause, often clustered and small, outbreaks of HCPS of which Andes (ANDV) seems the most widespread (332;333). Furthermore,

the world wide migration of rats resulted in the introduction of the Old World hantavirus Seoul (SEOV) in South America which is able to cause HFRS in human (334;335).

Currently, most of the diagnostics for (viral) infections are performed at the central laboratory of the Bureau of Public Health in Suriname. Starting from 2008, samples and clinical data have been stored from patients for which dengue, leptospirosis or influenza tests were negative, with the aim to use in future retrospective studies into the presence of (newly) emerging infectious diseases. Here we aimed to gain insight in the emergence of CHIKV in Suriname through retrospective analysis of samples collected in the period 2008-2012 and prospective analysis of samples collected in 2014. In addition we investigated the possibility of hantavirus infection in patients with no adequate diagnosis at the time of disease in Suriname.

Materials and methods

Study design and study site

This study was executed in Paramaribo, at the central laboratory, department of virology, from the Bureau of Public Health. Paramaribo is the capital and largest city of Suriname, located in the Northern part of the country at the banks of the Suriname river close to the North Atlantic coast. The laboratory is the major diagnostic centre in Suriname. A CHIKV and hantavirus retrospective serological study was conducted, followed by a prospective CHIKV serological and molecular surveillance study until we detected the first autochthonous CHIKV cases in Suriname in May and June 2014.

CHIKV serology

CHIKV IgM and IgG serology was tested by use of an in-house immune fluorescence assays (IFA) and commercial available IFA (Euroimmun, Germany). Serum samples were diluted 1:10 and 1:100. CHIKV diagnosis was confirmed in the laboratory of Erasmus MC, Rotterdam, the Netherlands which is a World Health Organization reference laboratory for arbovirus diagnostics.

CHIKV detection by real-time PCR

The presence of CHIKV in patient serum samples was determined by qRT-PCR, in the secondary laboratory in Rotterdam, the Netherlands, by detection of the E1 gene (336). Briefly, CHIKV RNA was extracted from 100 µl serum using the MagNAPure LC robot system and MagNA Pure LC Total Nucleic Acid Isolation kit (Roche, Germany). The presence of viral RNA was measured with a real-time qRT-PCR assay (TaqMan®Fast Virus 1-Step Master Mix, Invitrogen, Life Sciences) using Applied Biosystems® 7500 Real-Time PCR system.

CHIKV isolation and detection by electron microscopy

Patient sera, with a positive result in the CHIKV real-time PCR, were diluted 1:100 in medium and added onto a 70-80% monolayer of C6/36 insect cells and incubated for 60 minutes at 30 °C. The supernatant was discarded and cells were washed once and incubated with fresh Leibovitz-15 medium supplemented with 5% heat inactivated fetal bovine serum (HI-FBS), 10% tryptose phosphate broth, 0.75% sodium bicarbonate, 10 mM hepes buffer, 100 U penicillin, 100 µg/ml streptomycin and 2mM L-glutamine for an additional five days. All culture reagents were obtained from Lonza, Breda, The Netherlands. Cultures were incubated for five days. Supernatant from passage two was used for electron microscopy. To this end, 2 µl of cell free supernatant was pipetted on the Formvar carbon grids (SPI Supplies, USA) and air-dried at room temperature. Subsequently, dried grids were incubated for five seconds on parafilm containing one droplet of 2% phosphotungstic acid solution (pH 6.5). Grids were stored in ampules and examined with a Morgagni electron microscope at 80 kV.

Sequence and phylogenetic analysis of CHIKV E1 gene

A PCR targeting a portion of the envelope protein 1 (E1) gene was performed for one virus isolate. PCR products were separated on 1% agarose gel and bands of correct size were collected for DNA gel extraction using MinElute Gel Extraction Kit Protocol (Qiagen, U.S.A.). Amplicons were purified and sequenced using the Sanger method and the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI Prism 3130XL Genetic Analyser sequencer (both from Life Technologies). The phylogenetic tree was constructed using 800 nucleotides of the CHIKV E1 gene. Sequences were aligned using MUSCLE (v3.7) and the phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0). Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates). All software was used via <http://phylogeny.lirmm.fr/> as described in (337).

Hantavirus serology

Sera were screened using pan-hantavirus IgG and IgM DxSelect ELISAs (Focus Diagnostics, USA). These ELISAs are used for testing a broad range of hantaviruses, although there are variations in sensitivity and specificity for each specific hantavirus (338). According to the manufacturer's data both tests were compared with a reference in-house ELISA by external investigators. For Suriname the performance of these ELISA's in detecting New World hantaviruses and, potentially, Seoul hantavirus, is of importance. For New World hantavirus infections the IgM ELISA sensitivity is reported to be 100% in a small cohort (11/11 samples from Andes virus patients tested positive) and for the IgG ELISA this was 60% (3/5 samples from Andes virus patients tested positive). Based on SEOV FRNT-positive samples, the sensitivity was 50% (11.8–88.2%) in the IgM ELISA and 95% (54.1–100%) in the IgG ELISA (339).

ELISA IgM-positive or equivocal sera were tested in IFA by using commercial slides, Euroimmun hantavirus Mosaic – 1 (Euroimmun, Germany). This test is based on biochip slides coated with Puumala (PUUV), Seoul (SEOV), Dobrava (DOBV), Hantaan (HNVT), Saaremaa (SAAV) and Sin Nombre (SNV) antigen. Samples were pre-treated with Eurosorb IgG absorbent (Euroimmun) and subsequently tested in dilutions 1:10 and 1:100 according to the manufacturer's protocol.

Hantavirus focus reduction neutralization test (FRNT)

All samples positive in the pan-hantavirus IgM ELISA and Euroimmun MOSAIC IgM IFA were selected for FRNT confirmation. FRNTs for Seoul (SEOV) strain 80-39 and Andes (ANDV) strain Chile-9717869 were carried out as described elsewhere (317;338). Diluted sera were mixed with an equal volume of diluted virus containing 30–70 focus-forming units/100 µl. The serum end-dilution was 1:40. The mixture was incubated at 37 °C for 1 hour and subsequently inoculated into wells of six-well tissue culture plates containing confluent Vero E6 cell monolayers. The wells were overlaid with a mixture of agarose and tissue culture medium and incubated for 7–13 days. The agarose was removed from the wells and the cells were fixed. Antibody detection of SEOV and ANDV infected cells was done as described elsewhere (319). This step was followed by adding peroxidase-labelled conjugate followed by substrate reaction (tetramethylbenzidine) and foci were counted. An 80% reduction in the number of foci, compared with the virus control, was used as the criterion for virus neutralisation titres.

Results

Retrospective analysis of the presence of CHIKV antibodies

To test if CHIKV was already present in Suriname all samples sent to BOG in the period 2008-2012 of patients with symptoms of fever and arthralgia or fever and rash were retrospectively tested for the presence of CHIKV IgM or IgG by in-house and commercial IFA. In total 194 samples matched these criteria. Serum was available for 68 of these samples. All samples were non-reactive in the assays except one that showed low reactivity in the in-house developed CHIKV IgG IFA (positive in 1:10 dilution) drawn from a patient with a three day history of fever and rash, but no reactivity was seen in the in-house developed CHIKV IgM IFA or the commercially available IgM & IgG IFAs.

Prospective surveillance for the introduction of CHIKV in to Suriname

Starting from March 2014 CHIKV serological and molecular diagnosis was implemented in the diagnostic laboratory at BOG. Physicians in Suriname were informed about the possibility of CHIKV circulation and the availability of diagnostics. On average 8-10 samples were tested per week. The first cases testing positive by the IgM in-house developed IFA were observed in late spring (May 2014). All positive IFA results, and a random selection of the negatives, were sent for confirmation to a second laboratory. Serological results and clinical data for the first two

months of prospective CHIKV screening are listed in Table 1,. In total, 23 samples tested positive in Suriname of which the secondary laboratory confirmed ten samples. All negative test results were confirmed in the second laboratory. One sample (nr. 19) also tested positive by the RT-PCR for CHIKV. This sample was drawn from a patient presenting with arthralgia, haemorrhage (skin bleeding) and a macopapular rash over the whole body (Table 1).

Case	Date	Ethnicity	Age	Sex	IgM ¹	IgG ¹	IgM ²	IgG ²	PCR	Clinical information
1	May -14	Creole	25-29	M	neg	neg	neg	neg	neg	Fever, arthralgia, headache, myalgia, nausea
2 ¹	May-14	mixed	44-49	M	neg	neg	neg	neg	neg	Fever, Arthralgia, Weakness of limbs, Neck stiffness
4	May-14	mixed	30-34	M	≥100	neg	≥64	neg	neg	Fever, generalized pain, headache, dizziness, nausea, vomiting
5	June 14	Creole	55-59	F	neg	neg	neg	neg	neg	Fever, chills, conjunctivitis, diarrhea, dizziness, headache, malaise, myalgia, sore throat
6	June 14	Hindi	25-29	F	10	neg	16	neg	neg	Fever, generalized pain, rash (whole body)
7	June 14	unknown	25-29	F	neg	neg	neg	neg	neg	No additional information
8 ^{1,2}	June 14	mixed	60-64	F	neg	≥100	neg	≥64	neg	Fever, arthralgia, cough, dizziness, headache, malaise, neck stiffness, sore throat, vomiting, 4 weeks ago rash (whole body)
3	June 14	Asian	25-29	F	neg	neg	neg	neg	neg	Fever, persistent arthralgia, rash 4 weeks ago, dizziness, headache, malaise, nausea, vomiting
9 ³	June 14	Hindi	45-49	F	neg	neg	neg	neg	neg	Fever, arthralgia
10 ³	June 14	Hindi	45-49	M	neg	neg	neg	neg	neg	Fever, arthralgia, conjunctivitis, headache, rash (whole body)
11 ³	June 14	Hindi	10-14	F	neg	neg	neg	neg	neg	Fever, arthralgia, headache, rash (whole body)
12	June 14	mixed	15-19	M	≥100	neg	≥64	neg	neg	Fever, back pain, conjunctivitis, cough, diarrhea
13	June 14	Javanese	30-34	M	neg	neg	neg	neg	neg	Chills, rash (face), lower resp. complaints
14	June 14	Creole	30-34	M	≥100	neg	≥64	neg	neg	Fever, pain (generalized), rash (whole body), headache
15	June 14	Chinese	50-54	M	≥100	neg	≥64	neg	neg	Fever, pain (generalized), haemorrhage, malaise, myalgia, diarrhea
16	June 14	Javanese	40-44	M	≥100	neg	≥64	neg	neg	Fever, arthralgia, rash (whole body)
17	June 14	Hindi	20-24	F	≥100	neg	≥64	neg	neg	Fever, pain (generalized), rash (whole body)
18	June 14	Creole	15-19	F	10	neg	16	neg	neg	Fever, pain (generalized), cough, nausea, vomiting
19	June 14	mixed	30-34	F	10	neg	16	neg	CT 22.8	Fever, rash (whole body), arthralgia, haemorrhage, headache, malaise, myalgia, vomiting
20	June 14	mixed	65-69	F	neg	neg	neg	neg	neg	Fever, arthralgia, headache, neck stiffness, hemorrhage
21	June 14	Javanese	55-59	F	neg	neg	neg	neg	neg	Fever, arthralgia, haemorrhage, myalgia, retro-orbital pain, sore throat
22	June 14	Javanese	30-34	F	neg	10	neg	16	neg	Fever, haemorrhage, nausea, vomiting, myalgia, neck stiffness, rash
23	June 14	mixed	25-29	M	neg	neg	neg	neg	neg	Fever, pain (generalized)

Table 1: Results from prospective analysis of chikungunya cases in Suriname in May and June 2013. IFA = immunofluorescence assay, F = female, M = Male, Neg = Negative ¹ = travelled <15 to the Caribbean, ² = Sample is drawn >4 weeks since onset of the symptoms, ³ = originate from one family, IgM¹ & IgG¹ = the results from the in-house developed CHIKV IFA, IgM² & IgG² = the results from commercially available CHIKV IFA.

CHIKV virus isolation, sequence and phylogenetic analyses of the E1 gene and electron microscopy imaging

To confirm the molecular detection of CHIKV RNA in the serum of case number 19, this sample was cultured. Figure 1A shows the CT values of the inoculum, supernatant after passage number one and of the supernatant after passage number two. The rapid increase in viral RNA suggested replicating CHIKV. This was confirmed by use of electron microscopy. Microscopy of multiple samples drawn from the culture supernatant revealed particles of typical size and shape of that reported from CHIKV particles (Figure 1C&D). Phylogenetic analyses of the 800 nucleotides PCR product showed clustering of the Suriname isolate with isolates of the CHIKV Asian genotype. Closest relation was seen with the British Virgin Island isolate from 2014 (340).

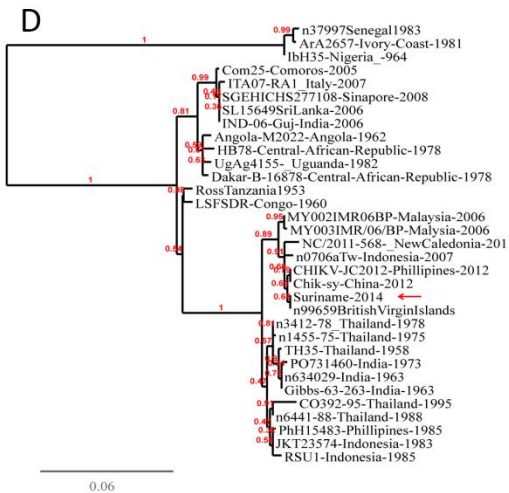
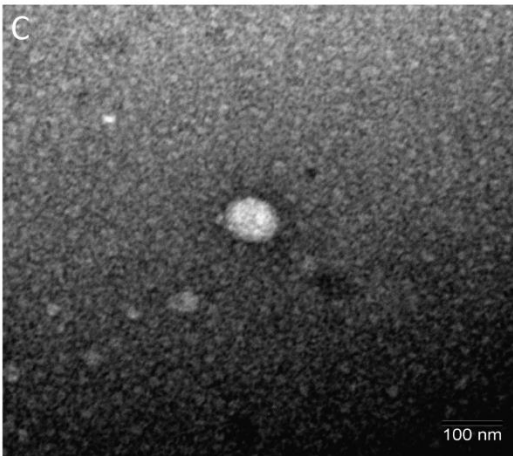
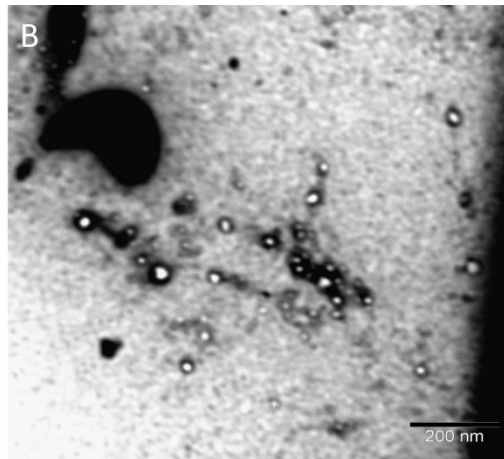
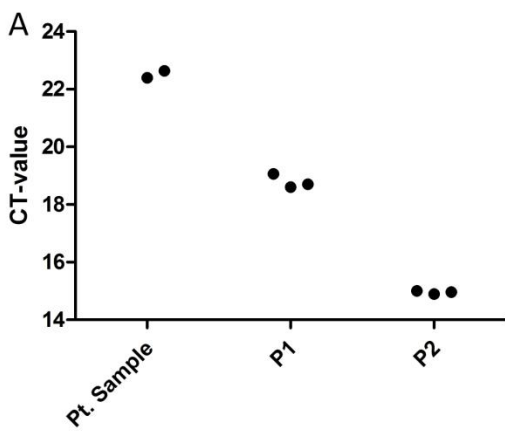


Figure 1. Panel A shows the decrease in CT value when sample from case number 19 was passaged over insect cells. Cultured virus was detected with electron microscopy, in panel B a cluster of virus particles is seen and in Panel C a single chikungunya virus particle in higher magnification. Panel D shows the result from phylogenetic analysis. The chikungunya isolate from Suriname is closely related to the strain isolated in the British Virgin Islands and seems to be of the Asian genotype.

Serological indication of human hantavirus infections in Suriname based on enzyme-linked immunosorbent assay and immunofluorescence assay results

In total 532 samples were submitted to BOG coming from patients suspected for leptospirosis or presenting with the combination of symptoms of fever and respiratory complaint or fever and signs of haemorrhage. All selected and available sera (n = 264) were screened by pan-hantavirus ELISA. Results are listed in Table 2. IgM testing resulted in 45 samples with an Optical Density (OD) of >1.1 times the cut-off calibrator, which, according to the manufacturer can be regarded as a positive result. Furthermore, five samples gave an equivocal result in the IgM ELISA (OD 0.9-1.1 times the cut-off calibrator). In total 18 samples tested positive for the presence of hantavirus IgG antibodies and two samples had an equivocal test result. Samples with a positive result in IgM or IgG ELISA were tested with IFA slides reactive with six different hantaviruses (PUUV, SEOV, HNTV, SNV, DOBV, & SAAV). Results from individual samples are listed in Table 2 together with specific clinical data and additional information. In total 21 of the 45 samples positive in IgM ELISA were also found positive by IFA testing. Reactivity against both Old and New World hantaviruses in IFA were found in multiple samples. Since recent published work has revealed numerous false positive samples in a comparable cohort study (338), we decided to repeat the initial ELISA screening by hands of a second investigator unaware of the first results. Twenty-five of the forty-five samples (56%) tested positive (OD of >1.1 times the cut off calibrator) in the second IgM ELISA. Second IgG ELISA analysis confirmed the presence of IgG hantavirus antibodies in 9 of these 18 samples (50%), while one equivocal sample tested negative and one positive (Table 2).

Confirmation by focus reduction neutralisation test (FRNT)

FRNT was performed on 21 sera with a positive IgM response in the hanta mosaic IFA. No samples showed significant neutralizing activity against SEOV or ANDV. Therefore, FRNT did not yield any evidence for infection with either SEOV or ANDV.

Sample Number	Date	ELISA				IFA MOSAIC	Sex	Race	Age Group in years	Information
		IgM ¹	IgM ²	IgG ¹	IgG ²	IgM	M/F			
47	Jan-08	2.4	1.2	NEG	NEG	SEOV	F	Creole	40-44	Fever
73	Feb-08	2.0	2.5	EQUI	EQUI	SEOV, SNV, DOBV, SAAV	F	Creole	25-29	Fever, Respiratory complaints, Chills, Sore throat, Abdominal pain, Headache
90	Feb-08	1.5	0.9	NEG	NEG	SEOV, DOBV, SAAV	F	Chinese	45-49	Fever, Chills, Cough, Headache, Myalgia, Haemorrhage
106	Feb-08	5.2	EQUI	1.5	1.1	ALL POS	F	Creole	>60	Fever, Chills, Headache, Myalgia, Sore throat, Vomiting
202	May-08	3	NEG	1.5	1.1	SEOV	M	Hindi	>60	Fever, Diarrhea, Haemorrhage, vomiting, thrombocytopenia
205	Jun-08	2.2	1.6	1.1	NEG	HNTV, SNV	F	Creole	5-9	Fever, Respiratory complaints, Abdominal pain, Cough, Chills, Vomiting
312	Aug-08	1.8	EQUI	NEG	NEG	SAAV	M	Creole	55-59	Fever, Back and abdominal pain, Headache
386	Oct-08	2.1	1.6	NEG	NEG	HNTV, SAAV	F	Dutch tourist	20-24	Fever (>40°C)
418	Nov-08	1.5	1.2	NEG	NEG	HNTV	M	Caucasian	>60	Fever, Haemorrhage
311	Mar-09	3.2	2.1	NEG	NEG	ALL POS	F	Hindi	0-4	Fever, Respiratory complaints
375	Mar-09	1.3	EQUI	EQUI	1.1	ALL POS	M	Creole	30-34	Fever, Respiratory complaints, Oedema, Severe dyspnea
403	Apr-09	EQUI	EQUI	2.4	2.2	ALL POS	F	Creole	>60	Fever, Respiratory complaints, Cough, Vomiting, Cardiac arrhythmia, Oedema
404	Apr-09	2.5	2.8	NEG	1.1	SNV, SEOV, SAAV	F	Creole	20-24	Fever, Respiratory complaints, Abdominal pain

426	Apr-09	2.2	NEG	NEG	NEG	SNV	F	Hindi	45-49	Fever, Respiratory complaints, Vomiting
749	Jun-090	1.4	EQUI	NEG	NEG	SAAV	M	Hindi	20-24	Fever, Vomiting, Lepto Rapid positive
1146	Aug-09	1.2	NEG	NEG	NEG	SNV	M	Creole	20-24	Fever, Sore throat, Nausea, Retro-orbitale pain, Rash, Haemorrhage
1449	Dec-09	1.9	1.2	NEG	NEG	PUUV, SAAV	M	Mixed	1-4	Fever, Respiratory complaints, Tachypneu, Rash
559	Jul-10	1.8	EQUI	NEG	NEG	SNV	F	Unknown	20-24	Fever, Respiratory complaints, Nausea, Vomiting
084	Jan-11	2.3	1.1	NEG	NEG	SEOV	F	Unknown	20-24	Fever, Respiratory complaints, Dizziness, Headache, Retro-orbital pain
223	Apr-11	NEG	NEG	2.8	1.7	DOBV, SEOV	M	Unknown	50-54	Fever, Cough
269	May-11	2.4	2.3	NEG	NEG	SEOV	F	Unknown	10-14	Fever, Jaundice, Necrotic wounds
374	Aug-11	3.8	3.0	NEG	NEG	SEOV, SAAV	F	Unknown	1-4	Fever, lymphadenopathy

Table 2. Hantavirus screening results. ELISA results are given in optical density (OD) ratio compared to the cut off calibrator. A result of >1.1 is considered positive while a result between 0.9 and 1.1 is considered as equivocal (EQUI). NEG = negative, M = Male, F = Female. IFA = Immune fluorescence assay for which an IFA test was used which is able to distinct between six hantaviruses being: SEOV = Seoul virus, SNV = Sin Nombre virus, SAAV = Saaremaa virus, HNTV = Hantaan virus, PUUV = Puumala virus. Only results positive in 1:100 dilution are given.

Discussion

This study reports the methods and data on the first detection of CHIKV in the republic of Suriname. Furthermore, our results indicated the occurrence of human hantavirus infections in Suriname. The introduction of CHIKV in Suriname follows the large outbreak in the Caribbean, following the first reports of introduction of CHIKV to the New World in December 2013 (342). With the magnitude of trade and travel between the Caribbean (the CHIKV outbreak areas) and Suriname, the risk of introduction of CHKV into Suriname was relatively high at forehand. Latest numbers from the national centre for statistics (Algemeen Bureau voor Statistiek Suriname; ABS) show that up to 8% of all people who enter Suriname originate from the Caribbean (343). Therefore, local authorities have put much effort in raising CHIKV awareness in (returning) travellers and health care workers in all areas of the country, putting special emphasis on the border areas with neighbouring countries. The fact that this eventually failed clearly shows the difficulties in preventing the spread of this emerging arthropod-borne virus. Analysis of the first cases in Suriname shows several interesting observations. First of all, the clinical signs and symptoms, as summarized in Table 1, include the classical symptoms of rash and arthralgia in almost all confirmed patients. Other symptoms include headache, myalgia, arthritis, conjunctivitis, vomiting, and maculopapular rash. Furthermore, in three out of the 11 confirmed CHIKV patients haemorrhage occurred, most often described as skin bleeding, conjunctival bleeding or nose bleedings. Although in our cohort this observation is based on a small number of patients, it does show the potency of haemorrhage in the course of infection of CHIKV. Something that could be validated by combining the clinical data from the Caribbean outbreak. Bleeding complications in CHIKV have been reported previously which raises the question if CHIKV should be categorized as a (mild) viral haemorrhagic fever pathogen (344-346). Currently, the Pan American Health organisation (PAHO) puts much effort in controlling and monitoring the CHIKV outbreak in the Caribbean and subsequently South America. The case definition used for CHIKV surveillance is formulated by PAHO and the WHO as follows: a suspected case is a person with fever $>38.5^{\circ}\text{C}$ and severe arthralgia/arthritis not explained by other medical conditions who is residing or has visited epidemic areas within 15 days prior to onset of symptoms. A confirmed CHIKV case is a suspected patient meeting laboratory confirmation criteria, which are either virus culture, RT-PCR, IgM antibody assay in single sample or fourfold increase in CHIKV specific antibody titres IgG (330). Potential problems in estimating the magnitude of the CHIKV outbreak in the Caribbean could be specificity and sensitivity of newly introduced diagnostics in centres not familiar with these procedures. In our small cohort, a large number of positive IgM IFA tests could not be confirmed in a secondary laboratory by gold standard serology testing. This illustrates the need for assay validation in epidemiological research, in order to get solid and trustworthy data. Reports depending on only IgM assays in one single sample should be interpreted with caution. Phylogenetic analysis of the E1 protein gene was in line with other reports coming from the Caribbean, indicating that

CHIKV circulating in Suriname is of Asian genotype, same as the virus detected on St. Martin island. The Asian genotype introduced in the Caribbean is closely related to strains recently isolated in China and the Philippines, suggesting that CHIKV strains circulating currently in the Western hemisphere originated from strains recently circulating between China, the Philippines and Yap in Southeast Asia (340). CHIKV disease is highly debilitating and can have severe economic consequences (347). Therefore, there is an urgent need for studies focusing on prevention, intervention (antiviral and vaccine developments), pathogenesis and treatment of CHIKV infection. Screening for hantavirus antibodies in undiagnosed patients in samples submitted to the B.O.G. diagnostic laboratory indicated the possibility of human hantavirus infections in Suriname. Based on the results and case definition from the Dutch Hanta-Hunting study, confirmation of a routine serological test by FRNT is needed to define a 'definite' hantavirus case. Since FRNT did not confirm the presence of (neutralizing) hantavirus antibodies in any of our samples we cannot draw a firm conclusion about the presence and magnitude of hantavirus infections in Suriname. The lack of confirmation of the serological screening is the major limitation of our study. However, the high number of 'probable' hantavirus cases as presented in Table 2, do stress the need for prospective studies addressing the presence and burden of hantavirus disease in Suriname. For confirmation by neutralization tests we relied on the hanta virus species for which stocks are available. For us this meant that FRNT could be performed with one New World hantavirus: ANDV and one Old-World hantavirus SEOV with a large global spread due to the global distribution of its vector the black and brown rats. New World ANDV is known to circulate in the Southern parts of South America and with the long tailed pygmy rice rat (*Oligoryzomys longicaudatus*) as its reservoir (98). Currently, no data are available of exact rodent species present in Suriname. Data from French Guiana, located on the eastern border of Suriname, showed the circulation and human cases of Maripa hantavirus infection, closely related to Rio Mamore hantavirus which circulates in Brazil (332). Furthermore, a recent publication described the circulation of both Araraquara and Juquitiba virus in a region in Brazil closely to the southern border of Suriname (331). Since pathogenic hantaviruses are (almost) always associated with one specific rodent species, different or even undiscovered hantaviruses might circulate in Suriname. This could explain positive results in routine serology due to cross-reactivity with hantavirus species in the panel, but lack of FRNT confirmation. Another option can be that both ELISA and IFA test generated false positive results. Potential limitation of routine diagnostic tests performed could have great implications for interpretation of epidemiological reports based on ELISA and IFA results only. Furthermore, the usability of these commercially available assays in areas where hantavirus epidemiology is not clearly studied could be debatable, with absence of FRNT confirmation. Especially the value of one positive result either in ELISA or IFA warrants further evaluation. Molecular detection of pathogenic hantaviruses either in rodents or symptomatic patients is needed to confirm the presence and circulation of hantaviruses in Suriname.

CHAPTER 3

PATHOGENESIS: “Clotting and haemorrhage in infectious diseases”

Consists of:

- 3.1** Effect of Puumala hantavirus infection on Human Umbilical Vein Endothelial Cell haemostatic function: Platelet interactions, increased Tissue Factor expression and fibrinolysis regulator release.
- 3.2** A comparative proteomic analysis of Human Umbilical Vein Endothelial Cells after infection with Puumala hantavirus and *Leptospira interrogans*, causative agents of rodent-borne haemorrhagic fevers
- 3.3** Leptospiruses and the activation of endothelial cells an *in vivo*, *in vitro*, *in vivo* approach.
- 3.4** Activation of coagulation and tissue fibrin deposition in experimental influenza in ferrets.

CHAPTER 3.1

Effect of Puumala hantavirus infection on Human Umbilical Vein Endothelial Cell haemostatic function: platelet interactions, increased tissue factor expression and fibrinolysis regulator release

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Abstract

“Puumala virus (PUUV) infection causes over 5000 cases of haemorrhagic fever in Europe annually and can influence the hemostatic balance extensively. Infection might lead to haemorrhage, while a recent study showed an increased risk of myocardial infarction during or shortly after PUUV infection. The mechanism by which this hantavirus influences the coagulation system remains unknown. Therefore we aimed to elucidate mechanisms explaining alterations seen in primary and secondary haemostasis during PUUV infection. By using low passage PUUV isolates to infect primary human umbilical vein endothelial cells (HUVEC) we were able to show alterations in the regulation of primary- and secondary haemostasis and in the release of fibrinolysis regulators. Our main finding was an activation of secondary haemostasis due to increased tissue factor expression leading to increased thrombin generation in a functional assay. Furthermore, we showed that during infection platelets adhered to HUVEC and subsequently specifically to PUUV virus particles. Infection of HUVEC with PUUV did not result in increased von Willebrand factor while they produced more plasminogen activator inhibitor type-1 (PAI-1) compared to controls. The PAI-1 produced in this model formed complexes with vitronectin. This is the first report that reveals a potential mechanism behind the pro-coagulant changes in PUUV patients, which could be the result of increased thrombin generation due to an increased tissue factor expression on endothelial cells during infection. Furthermore, we provide insight into the contribution of endothelial cell responses regarding haemostasis in PUUV pathogenesis.”

Introduction

Puumala virus (PUUV), a hantavirus carried by chronically infected bank voles, is the causative agent of an estimated 5000 cases yearly of viral haemorrhagic fever in Europe (307). Hantaviruses are rodent-borne, negative stranded, RNA viruses belonging to the *Bunyaviridae* family, which may cause two types of disease in humans (297). In Europe and Asia, hantavirus infection causes Haemorrhagic Fever with Renal Syndrome (HFRS), characterized by renal failure and bleeding complications. In North and South America, hantavirus infection causes the Hantavirus Cardiopulmonary Syndrome (HCPS) where patients present with severe acute respiratory distress (348). Changing ecological factors determine fluctuations in hantavirus epidemiology resulting in sudden increases in incidence, for instance through increased food availability, prolonged virus survival and decreased biodiversity (324). Recent epidemiological studies reported an overall incidence increase of PUUV infections in Europe (349).

Although PUUV infections have a low case fatality rate (<1%) and in literature the virus is often described as the least virulent of the pathogenic viruses within the hantavirus genus, PUUV infections can cause severe disease in healthy adults, which may require a long recovery period lasting up to one year (350). Furthermore, several reports described cases with severe (haemorrhagic) complications like pituitary gland haemorrhage, hematemesis and gastrointestinal bleedings (322;351). In contrast to these bleeding complications, a recent study from Sweden reported increased risk for acute myocardial infarction shortly after PUUV infection (352). Given the high incidence in Northern Europe, acute myocardial infarction as a complication of PUUV infection could have a major impact in endemic areas. In light of both bleeding and thrombotic events that might complicate PUUV infections, we hypothesized that endothelial cells, also the target cells for hantaviruses and the major regulators of coagulation and inflammation, play a central role in the pathogenesis of the disease (353).

During hantavirus infection drastic alterations in the coagulation system have been observed (354). Clinical studies focusing on primary and secondary haemostasis during hantavirus disease showed thrombocytopenia in both HFRS and HCPS, a decreased plasma activity of coagulation factors II, V, VIII, IX and X in acute HFRS patients, prolongation of the prothrombin and activated partial thromboplastin time, increased thrombin generation and D-dimer levels and a decrease in ADAMTS13 activity in acute PUUV patients (61-64). The ability to infect endothelial cells by hantaviruses has been demonstrated both *in vitro* and *in vivo* (355-357). Although infection does not lead to cytopathic changes, several studies observed endothelial cell dysfunction during hantavirus infection (356;357), ranging from increased clinical markers of a stressed endothelium *in vivo* (sICAM-1, VWF and circulating endothelial cells) (358;359), to increased permeability and decreased HUVEC integrin ligand migration *in vitro* (360;361).

Integrin $\alpha\beta3$, experimentally proven to be the receptor for hantavirus infection, is abundantly present on the surface of endothelial cells (362;363). Infection with pathogenic hantaviruses is suggested to result in the loss of function of the $\alpha\beta3$ integrin (364), but also an increased $\alpha\beta3$ expression on cultured endothelial cells and platelets has been observed (365). Furthermore Gavrilovskaya et al. studied the adherence of quiescent platelets to Sin Nombre and Hantaan virus infected endothelial cells seems to be the result of virus binding to the $\alpha\beta3$ integrin present on platelets (366).

How the abnormalities in the primary (thrombocytopenia) and secondary haemostasis (increase in thrombin generation and raised D-Dimer levels) are induced in PUUV infected patients and the mechanism by which Old-World hantaviruses cause haemorrhage and/or renal failure remain largely elusive (307). Lack of specific treatment and an effective vaccine makes understanding of the pathophysiology of hantavirus infection an important medical need, especially with the recently discovered association of PUUV with cardiovascular disease (352). Therefore, we have used an integrated approach to study changes in primary and secondary haemostasis using an *in vitro* endothelial cell model.

Methods

Cells

VeroE6 cells (American Type Culture Collection, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Lonza the Netherlands), 100 U/ml penicillin-streptomycin solution, 1% HEPES buffer and 1% sodium bicarbonate (all from Gibco, Life Sciences, USA). Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical veins, which were kindly provided by Erasmus MC birth center. Briefly, umbilical cords were stored in sterile 500 ml PBS supplemented with gentamycin (50 µg/ml) (Leo Pharmaceutical, Denmark). Veins were rinsed with PBS containing 50 U/ml heparin (Leo Pharmaceutical). Subsequently, cells were detached with 0.1% collagenase solution (C6885, Sigma Aldrich, USA). Cell suspension was collected in a sterile 50 ml tube followed by two times centrifugation (5 minutes 300 g). The cell pellet was re-suspended in HUVEC medium (human endothelial-SFM medium) (Invitrogen, Life Sciences, United States) containing 10% human serum (Lonza), 20% filtered FBS (Lonza); penicillin/streptomycin 100 U/ml, 20 ng/ml fibroblast growth factor (Peprotech, United States) and 10 ng/ml of endothelial cell growth factor (Peprotech). HUVEC cell suspensions were cultured in flasks pre-coated with 20 µg/ml of fibronectin (Roche, the Netherlands). Only cells up to passage four, from one specific donor, were used for this study. Identity of the endothelial cells was confirmed by flow cytometry using *Ulex europaeus* lectin, anti-CD31 and Von Willebrand Factor (VWF) staining and immunoblot.

Anti-sera

We made use of the following antibodies and conjugates: polyclonal rabbit anti- VWF, HRP labelled polyclonal goat anti-rabbit IgG and polyclonal rabbit anti-mouse (All from Dako, the Netherlands). FITC labelled monoclonal anti-CD31 (Sigma Aldrich, USA), polyclonal rabbit anti-CD41 (Perbio Science, the Netherlands), polyclonal rabbit anti-CD3 (Dako), polyclonal rabbit anti-PUUV nucleoprotein (BEI Resources, USA), monoclonal anti-PUUV glycoprotein (HY Test, Finland), monoclonal anti- $\alpha\beta$ 3 integrin (Abcam, UK), monoclonal anti-vitronectin (Novus Bio, USA), polyclonal rabbit-anti PAI-1 (Bio Connect, the Netherlands), polyclonal rabbit anti-tissue factor (Bio Connect) human serum from a recovered PUUV case described in (308). Antibodies and conjugate were diluted in dilution buffer, which consisted of PBS with 0.5% bovine serum albumin, 2% NaCl and 1% normal goat serum.

Virus isolation

Lungs of *Myodes glareolus* from Konnevesi, Finland, infected with PUUV were homogenized in DMEM (10% w/v) and 100 µl was added onto a 70-80% monolayer of VeroE6 cells and incubated for 60 minutes at 37 °C in 5% CO₂. The supernatant was discarded and cells were

washed three times and incubated with fresh veroE6 medium for an additional five days. Virus stocks up to passage four were created by centrifugation (10 min. 400 g) of the supernatant to create a cell free virus stock. Virus titre was determined using immune peroxidase reaction (IPOX) and TCID₅₀ was calculated using the Karber formula (367). Infectious virus was inactivated using beta-propiolactone (BPL) (Sigma Aldrich, USA; 1:4000 v/v) at 4 °C for 24 hours. Subsequently, BPL was inactivated for one hour at 37 °C. All virus stocks were stored at -80 degrees until use. Vesicular stomatitis virus (VSV) strain Indiana, propagated also on VeroE6 cells, was kindly provided by Dr. Bart Haagmans (Erasmus MC).

Infection kinetics and dynamics

HUVEC were seeded into 24-well- (2.4×10^5 cells) or 96-well plates (4×10^4 cells) (Corning, USA) depending on the experiment. Confluent monolayers were infected with a multiplicity of infection (MOI) of 0.5 or 3, with infectious and inactivated (BPL-inactivated) virus or a normal medium control for 60 minutes at 37 °C in 5% CO₂. After incubation, the supernatant was discarded and cells were washed three times with RPMI 1640 (Gibco, Life Sciences). Fresh medium was added as described earlier. For VWF and plasminogen activator inhibitor type-1 (PAI-1) quantification, medium did not contain FCS but was supplemented with 4% sterile filtered bovine serum albumin (BSA, Sigma Aldrich, USA) to avoid addition of fetal calf VWF and PAI-1 (368). To quantify the percentage of infected cells we used an in house developed IPOX procedure. HUVEC were washed three times with PBS. Cells were fixed with absolute -20°C methanol and incubated at -20°C for 30 minutes. After fixation, methanol was discarded and cells were incubated for 30 minutes at 37 °C with 100 µl of 0.05% H₂O₂ in PBS, to block endogenous peroxidases. Subsequently, cells were washed three times with PBS and incubated for 60 minutes with polyclonal rabbit anti-PUUV nucleoprotein antibody (1:500). Cells were washed with PBS 0.05% tween followed by incubation with HRP-labelled goat anti-rabbit IgG conjugate (1:500). Colour development was achieved by addition of 3-amino-9-ethylcarbazole (AEC) substrate (AEC dissolved in dimethylformamide buffered with acetate buffer of pH 5). Percentage of infected cells was determined by manual counting.

For the quantification of viral replication we used a standard line of in house generated PUUV RNA run-off transcripts, as described for West-Nile virus (369). Briefly, RNA run-off transcripts were generated using a segment amplified with pan-hantavirus degenerative PCR primers from (370). PCR products were separated on 1% agarose gel and bands of correct size were collected for DNA gel extraction using the MinElute Gel Extraction Kit Protocol (Qiagen, United States). DNA fragments were cloned into the pCR4 vector using the TOPO® TA Cloning KIT (Life Technologies) and One Shot® TOP10 chemically competent *E.coli* were transformed with the recombinant vector (QIAGEN) according to manufacturer's protocol. At least five colonies were collected for further analyses. Plasmid DNA was purified using MinElute DNA purification kit (QIAGEN). Plasmid DNA was linearized by restriction digestion (NotI for the

negative strand RNA and PstI for the positive strand RNA). Run-off transcripts (in vitro transcripts) were synthesized using T3 RNA polymerase for negative strand and T7 RNA polymerase for positive strand (MEGAscript® T3 and T7 transcription kits, Life technologies), followed by DNase treatment (Ambion® TURBO DNA-free™ Life Technologies), according to manufacturer's manual. The amount of RNA in the stock was determined using NanoDrop® and serially diluted. Copy numbers in the standards were calculated using RNA concentration and sequence length with help of an online calculator(<http://endmemo.com/bio/dnacopynum.php>).

Platelet collection

To study interaction between PUUV and platelets, platelets were collected according to the protocol described in (366) with minor modifications. Briefly, blood was collected in 0.105 M (end concentration) sodium citrate tubes (BD-plymouth, UK) supplemented with 1 µM prostaglandin E1 (Cayman Chemical, USA) to block platelet activation. Platelet-rich plasma (PRP) was prepared by centrifugation for 15 min at 700 × *g* at 25°C. Subsequently, platelets were pelleted for 15 min at 1300 × *g* at 25°C. Platelets were washed twice and resuspended with modified hepes buffer (25 mM HEPES, 137 mM NaCl, 0.1% Albumin and 1 µM prostaglandin E1 pH 7.4) and counted by a hemacytometer.

Platelet HUVEC binding

Platelets (10⁸ per ml) were incubated with infected HUVEC (96 wells plate) (PUUV, BPL inactivated PUUV or mock control) for 30 minutes at 37°C. After incubation, monolayers were washed three times with RPMI and cells were fixed with formalin. After fixation, formalin was discarded and cells were incubated for 30 minutes at 37°C/5% CO₂ with PBS 0.05% H₂O₂. After three washing steps cells were incubated with rabbit polyclonal anti-human CD41a antibody (1:500). The following steps were as described earlier for IPOX. After incubation with HRP-labelled goat anti-rabbit conjugate (1:1000) TMB was added to the wells for substrate reaction. After 10 minutes reaction was stopped by addition of 0.5 M sulphuric acid and optical density (OD) was measured at 450 nm using Tecan ELISA reader. CD41a expression OD was calculated by subtracting the blank OD value (wells incubated without platelets but with detection antibody and conjugate). Rabbit polyclonal anti-CD3 (1:500) served as an isotype control.

Platelet PUUV binding

To test if changes in CD41a expression was related to direct binding between PUUV and platelets, a mechanism shown in Hantaan and Andes virus infection (366), a pull down assay was designed. To this end, we first coated ELISA plates with PUUV or a control virus (VSV) (100µl of 10⁶ virus particles in DMEM at 4 °C overnight) followed by platelet incubation (10⁷ platelets). Subsequently, cells were washed five times with PBS and the bound platelets were quantified by using a platelet detection antibody (anti-CD41a 1:500 in dilution buffer) followed

by a conjugate substrate reaction. CD41a expression was calculated by subtraction of the OD measured in the wells without platelet incubations (blanco) to correct for direct (a-specific) anti-CD41a antibody binding to PUUV and anti-CD3 was used as isotype control. Subsequently, ELISA plates were coated with mouse monoclonal anti-PUUV glycoprotein- or isotype control antibody (IgG2 corona virus) (1:500 in PBS at 4 °C overnight) followed by incubation with PUUV to capture the virus followed by platelet incubation, detection antibody and conjugate substrate reaction. Thirdly, to further confirm platelet PUUV binding, ELISA plates were coated with an anti-platelet antibody (1:500 in PBS at 4 °C overnight), followed by incubation with fresh isolated platelets and eventually an incubation step with PUUV or VSV followed by a hantavirus detection antibody (mouse mAB anti-PUUV-glycoprotein 1:500). After washing substrate reaction was achieved by conjugate addition and TMB reaction steps. As a final step we studied the potential blocking of platelet binding by PUUV particles by the addition of a blocking step with polyclonal human anti PUUV serum. PUUV coated plates and plates coated with 5 day old virus free VeroE6 medium were incubated with a polyclonal PUUV serum (1:50) from a case described in (308) or with a PUUV IgG negative control human serum from a healthy volunteer (also 1:50). The following platelet binding steps and CD41 detection were the same as in the earlier experiments.

Von Willebrand factor and plasminogen activator inhibitor type-1 quantification

After infection, in a 24-well plate, supernatants (500 µl) were subsequently removed and cells were lysed, after three washing steps, using a 15 minute incubation with 500 µl PBS 1% TritonX-100 followed by centrifugation (10 min. 400 x g). Cell-free supernatants and supernatant from cell lysates were measured using a PAI-1 antigen and VWF ELISA kits according the manufacturer's instructions (both from Zymugen, Hyphen Biomed, France).

Tissue factor cell surface expression

HUVEC in 96 wells plates were fixed with 4% formalin and incubated with rabbit polyclonal anti-tissue factor antibody (1:500) followed by incubation with the respective conjugate (1:500). After washing TMB was added for substrate reaction and reaction was stopped after 10 minutes by addition of 0.5 M of sulphuric acid. OD 450 nm value was measured on Tecan ELISA reader.

Tissue factor cell lysate concentration

Cell lysates were prepared as described under 2.5. ELISA plates were coated with a mixture of 50 µl cell lysate and 50 µl PBS over night at 4 °C together with a standard curve of recombinant tissue factor (Innovin; Siemens Healthcare Diagnostics, Germany). After blocking wells were incubated with rabbit polyclonal anti-tissue factor antibody (1:500) followed by incubation with the respective conjugate (1:500). After washing TMB was added for substrate reaction and

reaction was stopped after 10 minutes by addition of 0.5 M of sulphuric acid. OD 450 nm value was measured on Tecan ELISA reader.

Thrombin generation

Thrombin generation in platelet-poor plasma (PPP) was measured directly on HUVEC surface, in a 96 well plate, by recalcification of 80 μ l of pooled citrated plasma from healthy donors added to the monolayer of infected and uninfected cells. In summary, cells were washed three times with RPMI and 80 μ l freshly thawed plasma was added to the monolayer together with 60 μ l of HEPES buffer (25 mM HEPES, 137 mM NaCl, 0.1% albumin). On the same plate a serial dilution of recombinant tissue factor (TF) (Innovin; Siemens Healthcare Diagnostics, Germany) in the absence of cells. Finally, 60 μ l of HEPES calcium (25 mM HEPES, 137 mM NaCl, 0.1% Albumin, 38 mM CaCl₂) was added to plasma. Directly after recalcification, OD 450 nm value was measured using a Tecan ELISA reader in a kinetic cycle measuring every 45 seconds for 1 hour. Thrombin generation time was defined as the time at half-maximal OD.

Vitronectin – PAI-1 complex levels

ELISA plates were coated with anti-vitronectin antibody (1:500 in PBS at 4 C° overnight), incubated with supernatant from PUUV infected or non-infected HUVEC followed by incubation with polyclonal anti-PAI-1 antibody (1:500) and subsequent conjugate-substrate reaction. PBS incubation was used as a blanc control and anti-CD3 antibody (1:500) incubation as an isotype control.

Statistics

All statistical analyses were performed using GraphPad Prism 5.01 for Windows. When comparing two groups we made use of a Student's *t* test or Mann Whitney U, depending on the distribution of the data. For the comparison between multiple groups non-parametric Kruskal-Wallis test was used with Dunn's multiple comparison test or a one-way ANOVA with Tukey's multiple comparisons test, depending on the distribution of the data. *P* values ≤ 0.05 were considered significant.

Results

PUUV infects and replicates in primary endothelial cells

To prevent PUUV from *in vitro* loss of virulence, virus stocks of not more than four passages were prepared. Freshly isolated HUVEC were infected with MOI 0.5 and 3. The PUUV infected and replicated in HUVEC, as is summarized in Figure 1. Non-infected cells (Figure 1A, B) showed no red peroxidase staining, confirming specificity of the PUUV-staining. From 24 hours post infection with a low MOI infection (0.5) onward (Figure 1C) only a small percentage (+/-10%) of the cells were infected, which strongly increased after 48 hours (figure 1D), resulting in 50% of infected stained cells. Twenty-four hours after infection at a MOI of 3 about 40%-50% of cells were infected (Figure 1E), which increased further to 80% by 48 hours (Figure 1F). Comparable kinetics were seen when viral RNA copy numbers were determined. To this end, viral RNA numbers were estimated both in supernatant (Figure 1G) and cell lysate (Figure 1H). At both MOIs the number of viral RNA increased significantly (2-Log) after 48 hours (Kruskal-Wallis; $p = 0.0028$), in the supernatant as well as in the cell lysate, confirming active viral replication. Viral replication reached a plateau at 72 hours post infection. Furthermore western-blot analysis of the cell lysate for PUUV nucleoprotein confirmed infection of HUVEC (Figure 1I). The viral copy numbers in the supernatant or cell lysate of HUVEC incubated with BPL inactivated virus served as a control for non-replicating virus. Consistently, the RNA copy numbers did not increase over time indicating the efficient inactivation of the virus by BPL treatment. Efficient inactivation was confirmed by negative IPOX staining of the HUVEC incubated with BPL inactivated PUUV (data not shown).

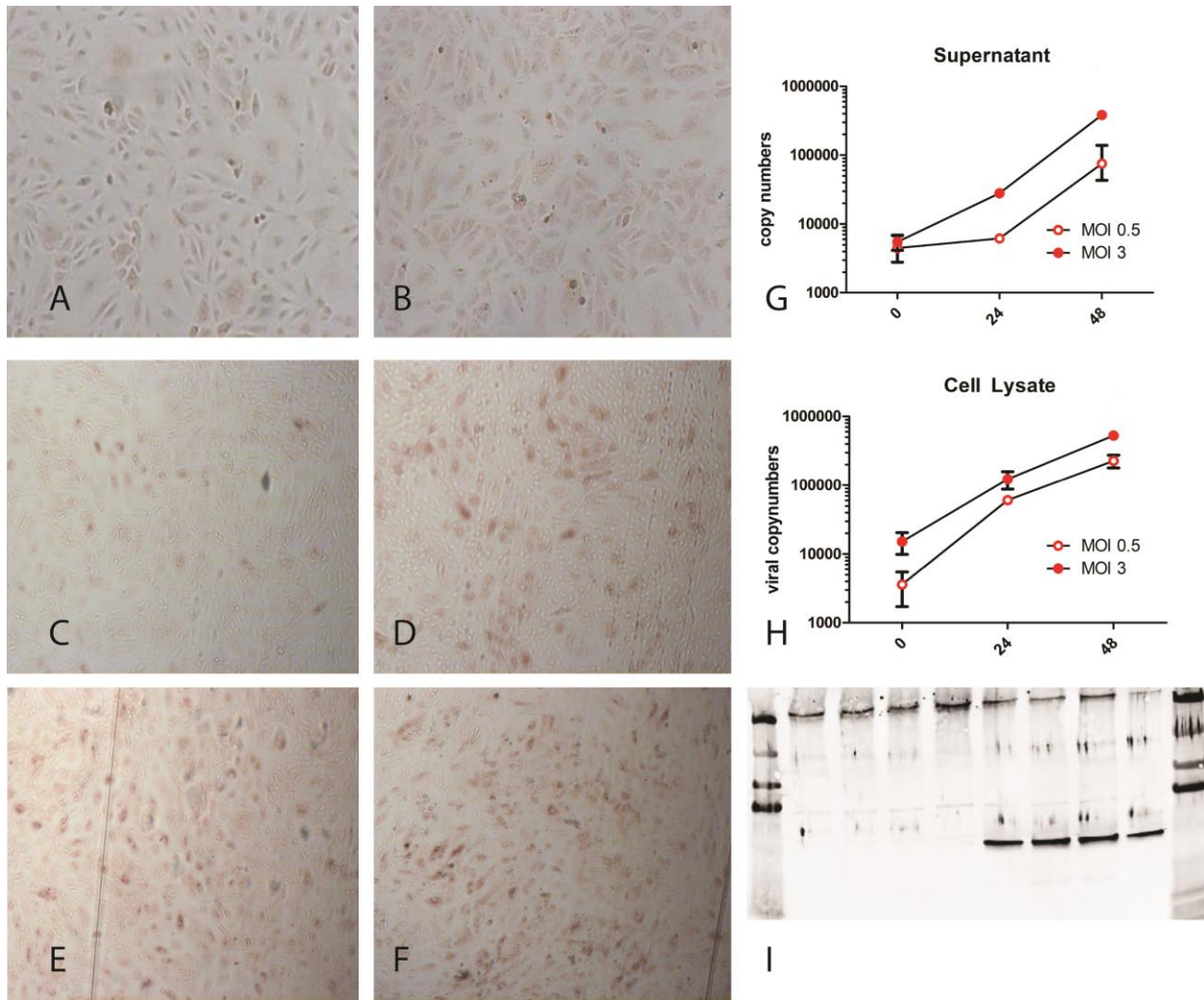


Figure 1. PUUV immune peroxidase (IPOX) staining and viral copy numbers after infection with a multiplicity of infection (MOI) of 0.5 and 3.

Mock infected cells (A&B) showed no red peroxidase staining for PUUV nucleoprotein after 24 (A) and 48 (B) hours. Twenty-four hours after infection with MOI 0.5 (C) a small number of cells stained positive, which, increased at 48 hours (D). Infection with MOI 3 resulted in more infected cells after 24 hours (E), which increased slightly at 48 hours post infection (F). Analysis of viral replication showed a more than 2-log increase of viral copy numbers in both supernatant (G) and cell lysates (H), suggesting active viral replication. Bars represent standard error of the mean. BPL inactivation of the virus lead to no increase in viral copy numbers in the supernatant and a negative IPOX staining (data not shown). Furthermore infection was confirmed by western blot for the presence of the PUUV nucleoprotein in the cell lysate and Von Willebrand Factor to confirm the character of the endothelial cells (I). The first four lanes show control wells with only one band present at the upper side of the blot (VWF). The last for lanes show the presence of both VWF and the PUUV nucleoprotein (approx. 55kDa 10ug/lane).

Increased CD41a expression after incubation of platelets on HUVEC upon PUUV infection

Gavrilovskaya and colleagues reported the potential of Hantaan and Andes virus to bind quiescent platelets via $\alpha\beta 3$ integrin (366). Since this observation is of much importance in further understanding the alterations in primary haemostasis and its role in disease mechanisms in HFRS we decided to confirm this mechanism for PUUV using a different approach. First we assessed the ability of quiescent platelets to bind to PUUV infected HUVEC (Figure 2). Binding of platelets was determined by measuring the intensity of CD41a (platelet glycoprotein IIb), a heterodimeric integral membrane protein present only on platelets and megakaryocytes. CD41a expression was significantly higher on the HUVEC monolayer after infection with a MOI of 0.5, or 3 compared to the control. Detection of CD41a expression did not differ when platelets were not added to the HUVEC monolayers, suggesting that there was no non-specific anti-CD41a binding to infected cells. Furthermore there was no difference in OD values when an isotype control (anti-CD3) was used to detect platelets. Based on CD41a expression, statistically significant differences were measured between infected wells and wells incubated with virus free VeroE6 medium (negative control) at 24 hours post infection (one way ANOVA, MOI 3 vs. NEG $p < 0.01$, MOI 0.5 vs Neg $p < 0.05$). After 48 hours of infection this difference in CD41a expression was also significant between MOI 3 infected wells and the BPL inactivated virus control (one way ANOVA, MOI 3 vs. NEG $p < 0.001$ and MOI 3 vs. BPL $p < 0.001$; MOI 0.5 vs. NEG $p < 0.05$). Taken together, the data indicate that platelets bind to cultures incubated with PUUV. HUVEC incubated with BPL did show a trend to increased platelet CD41a expression (Figure 2), but this was not statistically significant.

Von Willebrand factor (VWF) is not increased during PUUV infection of HUVEC

We measured VWF antigen in cell free supernatant and VWF expression on the surface of infected HUVEC. Increased VWF production may be a general inflammatory response of endothelial cells that could be evoked as a result of PUUV infection. However, at the time points where platelet binding increased, HUVEC infected with PUUV showed no alteration in VWF activity, as determined by ELISA, in neither the supernatant nor the cell lysate (Figure 3) compared to BPL or negative control.

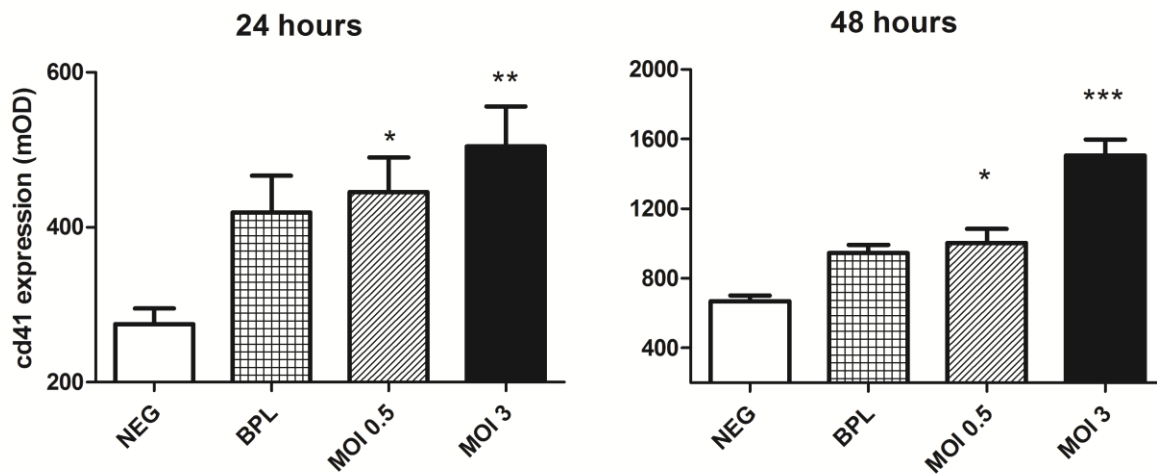


Figure 2. Increased platelet binding to PUUV infected HUVEC.

Increased optical density (OD) of CD41a was measured both 24 (A) and 48 (B) hours after infection with both low and moderate MOI and platelet incubation on HUVEC surface. P values (* $p < 0.05$ ** $p < 0.01$ *** $p = <0.001$) are the result of one way ANOVA testing with Tuckey's multiple comparison posttest. Bars represent the standard error of the mean. Incubation with an isotype control antibody (polyclonal anti-CD3) did not lead to increased OD on infected or control HUVEC.

Platelets bind directly to PUUV

Next we looked whether the platelets could bind directly to PUUV particles. For this purpose we performed an in-house developed platelet pull down-assay using quiescent platelets. To this end, several experiments were conducted to demonstrate specificity of this binding. Figure 4 shows results of binding of platelets to virus-coated ELISA plates (Figure 4 panel A). More platelets (Mann-Whitney U; $p=0.0022$) adhered to plates directly coated with PUUV compared to plates coated with a virus control (VSV), which was cultured under the same conditions as PUUV.

Subsequently, to control if the binding of platelets was directly to the PUUV particles and not due to another factor present in the VeroE6 supernatant we made use of a sandwich ELISA principle. PUUV was incubated on ELISA plates with wells coated with a monoclonal IgG2 specific for the glycoprotein of PUUV or with a IgG2 control antibody. By this approach significantly more platelets bound to the wells where PUUV was captured compared to wells with no PUUV capture (Mann-Whitney U ; $p = 0.0022$) (Figure 4B).

To confirm direct binding between platelets and PUUV, platelets were captured to anti-CD41 coated ELISA plates and incubated with virus followed by detection with a PUUV specific antibody. To control for binding between PUUV detection antibody and captured platelets, control wells were incubated with VSV. PUUV detection was significantly higher in the wells

incubated with PUUV compared to VSV (Mann-Whitney U ; $p = 0.0043$) (Figure 4C). These experiments collectively suggest that platelets can specifically bind to PUUV.

Finally, we show in Figure 4D that the binding of platelets to PUUV particles could be blocked by addition of a blocking step with human serum from a recovered PUUV case. When wells coated with PUUV were incubated with human serum with proven PUUV neutralizing IgG antibodies significantly less platelets adhered to the wells compared to wells incubated with a PUUV negative human control serum (Figure 4D).

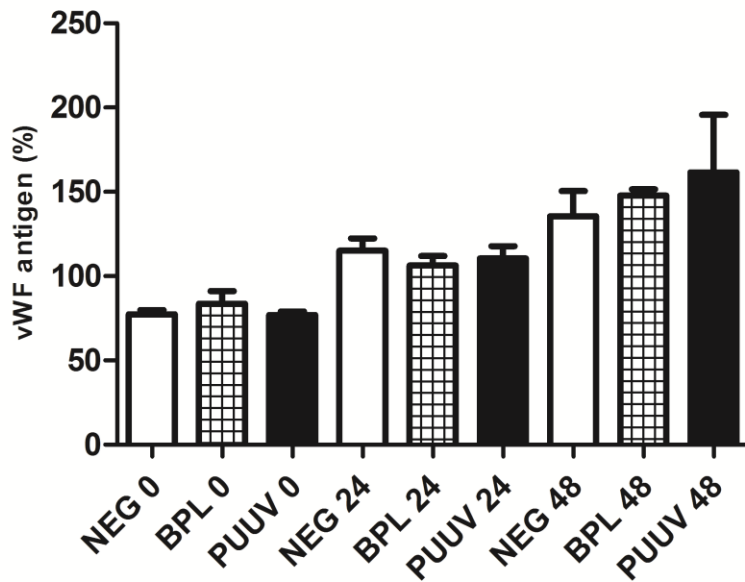


Figure 3. Von Willebrand factor (VWF) in cell free supernatant.

No increased VWF antigen is seen in the cell free supernatant of PUUV infects HUVEC in comparison to non-infected (NEG) and inactivated virus control (BPL) infected HUVEC. Data are representative of three independent experiments.

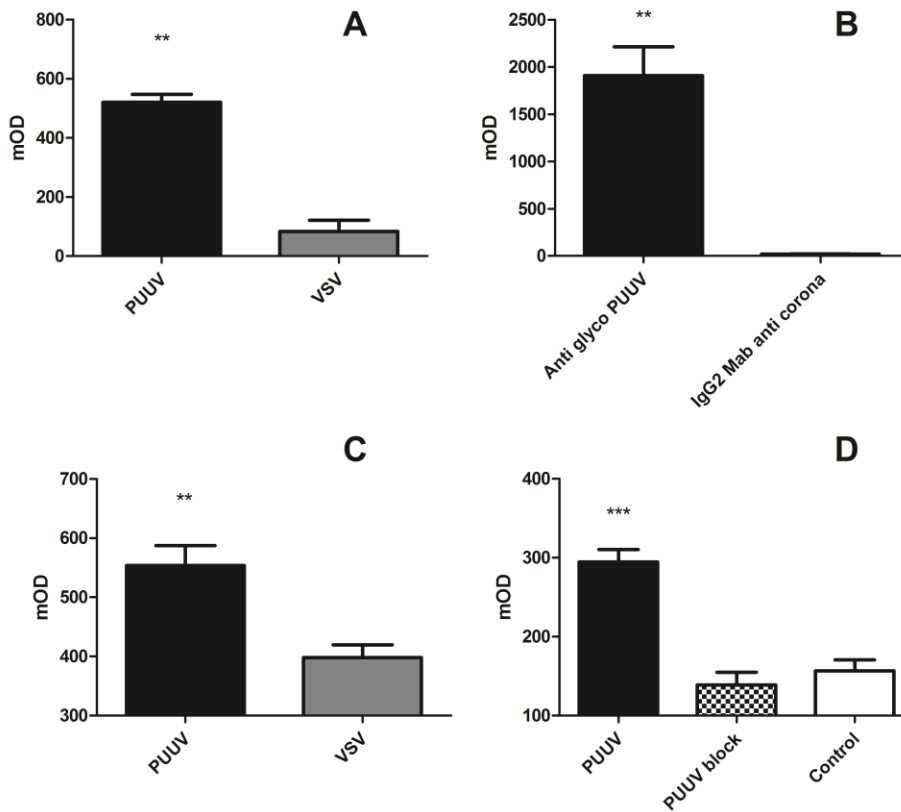


Figure 4. PUUV and platelets bind to each other

In a pull down assay platelets adhere better to PUUV virus particles compared to vesicular stomatitis virus (VSV) (A) (Mann Whitney U $p = 0.0022$). When virus was captured with a PUUV glycoprotein antibody (B), platelets were able to bind to the captured virus, in contrast to wells coated with an IgG2 control antibody (anti-coronavirus glycoprotein) (Mann Whitney U $p = 0.0022$), resulting in no capture of PUUV during the incubation process, controlling for potential other factors present in the virus stock medium. When platelets were bound to plates coated with an anti-CD41a antibody (C), the PUUV particles were able to bind to platelets based on the significant increase in PUUV detection OD compared to wells incubated with VSV particles, thus no PUUV present (Mann Whitney U $p = 0.0043$). The binding between PUUV and platelets could be blocked by the addition of a blocking step with human anti-PUUV serum (D) which show a decreased CD41 expression when compared to the PUUV coated wells incubated with a PUUV negative control serum. In all experiments no difference in OD was measured when an isotype control antibody was used. Data are representative of three independent experiments.

Increased thrombin generation and tissue factor expression after PUUV infection of HUVEC

To test the hypothesis whether increased thrombin generation observed in acute PUUV patients is the result of increased TF expression on endothelial cells we incubated HUVEC, infected with PUUV at a MOI of 3 or with a virus free five day old Vero E6 medium (control) with a polyclonal anti-TF antibody. By this approach we showed that TF expression was significantly increased with an almost two-fold increase in OD value 48 hours post infection (Figure 5A, Mann Whitney U; $p = 0.0047$). Cells infected with PUUV also showed an increased TF concentration when the cell lysates of PUUV infected wells were compared to the lysates of control wells (both mock and BPL). Subsequently we wanted to prove that the increase in TF expression on the endothelial cell surface was of biological significance and would lead to increased thrombin generation. Thrombin generation was quantified directly on infected endothelial cells by incubating normal plasma on cells and initiating coagulation by the addition of calcium ions. Infected cells induced plasma clotting faster due to increased thrombin generation (Figure 5 panel B & C). Using a calibration curve with purified TF in the absence of endothelial cells, we quantified HUVEC TF production after virus infection and after incubation with a virus free medium control. TF concentration showed a statistically significant increase for MOI 3 at 24 hours compared to the negative control and the HUVEC infected with MOI 0.5 (one way ANOVA; $p < 0.01$) and at 48 hours post infection compared to the negative control (one way ANOVA; $p < 0.001$). The MOI 0.5 infection led to higher levels of TF on the HUVEC surface only after 48 hours post infection (one way ANOVA; $p < 0.05$) when compared to mock.

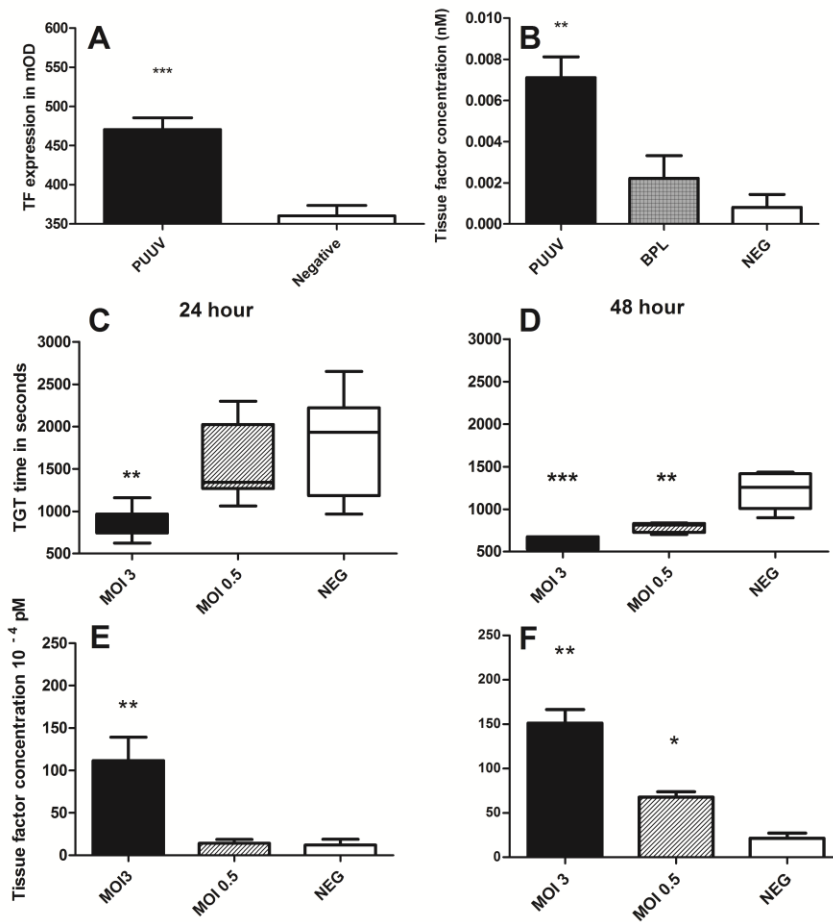


Figure 5. PUUV infection of HUVEC induces tissue factor expression resulting in enhanced thrombin generation

MOI 3 infection resulted in increased TF expression (A, Mann Whitney U $p = 0.0002$) on HUVEC surface after 48 hours (A) and in the cell lysate (B). Thrombin generation time (TGT) was significantly decreased, indicating more thrombin formation, for cells infected with PUUV (MOI 3) at 24 (C) and 48 (D) hours post infection (one way ANOVA; 24 hr $p < 0.01$ & 48 hr $p < 0.001$). Results are shown with whiskers from minimum to maximum. MOI 0.5 infection resulted in shortened TGT 48 hours post infection (one way ANOVA $p < 0.001$). Mean tissue factor (TF) concentration, calculated from TGT standard curve, increased more than 9-fold after MOI 3 infection (one way ANOVA 24 & 48h $p < 0.01$). MOI 0.5 infection increased TF concentration 3-fold after 48 hours (one way ANOVA $p < 0.05$) (E & F; bars represent standard error of the mean). Data are representative of three independent experiments.

Increased levels of PAI-1 and PAI-1-vitronectin complexes in HUVEC supernatant after infection with PUUV

Important proteins in the regulation of fibrinolysis show close interactions with the pathogenic hantavirus receptor $\alpha\beta 3$ integrin. For instance vitronectin, a stabilizer of plasminogen activator type-1 (PAI-1) activity in plasma, is largely regulated by this receptor (62;371). To study potential changes in regulators of fibrinolysis we first measured PAI-1 levels in the cell-free supernatant and supernatant of cell lysate from 24-well plates infected with PUUV or control infections. The total PAI-1 antigen (the combination of levels in the supernatant and cell lysate) was significantly increased 48 hours post infection (Kruskal-Wallis; $p < 0.05$) with MOI 3 (Figure 6A). Subsequently, we tested if in our model PAI-1 would bind to vitronectin, since this binding is associated with increased/prolonged PAI-1 activity (372), and if this interaction is altered during infection. ELISA plates coated with a monoclonal antibody against vitronectin, incubated with supernatant from our experiments (pooled, control or from MOI 3 infected wells) followed by incubation with PAI-1 antibody suggested formation of PAI-1 vitronectin complexes due to an increase in optical density compared to incubation with PBS (mean expression in medium 490 mOD (+/- 100) versus 370 mOD (+/- 70) $p = 0.02$). If supernatants were tested separately (PUUV versus mock) levels of PAI-1 vitronectin complexes were increased after PUUV infection (Mann-Whitney U; $p = 0.03$) (Figure 6B).

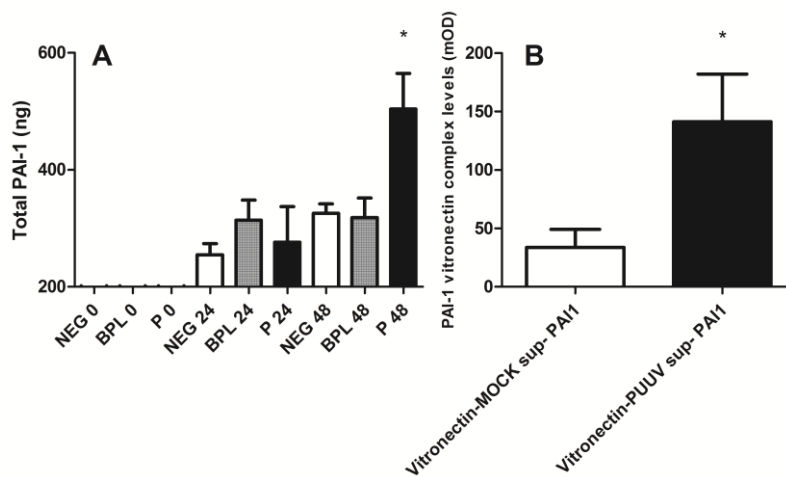


Figure 6. Plasminogen activator inhibitor type 1 production and PAI-1 vitronectin complex formation in HUVEC infected with PUUV.

Total plasminogen activator inhibitor type-1 (PAI-1) concentration was significantly increased on time points 48 post PUUV

infection (black) (one way ANOVA, $p < 0.05$) compared to BPL inactivated control (grey) or negative medium control (white). Panel B shows the PAI-1 vitronectin complex levels in HUVEC infected with PUUV (black) or non-infected controls (white). After 48 hours of infection PAI-1 vitronectin levels were increased compared to the mock infection (Mann Whitney U $p = 0.03$) (B). Data are representative of three independent experiments.

Discussion

The present study addresses platelet binding to PUUV infected cells and activation of secondary haemostasis after endothelial cell PUUV infection. With the lack of a valid and accessible animal model for old-world hantavirus infection, we remain dependent on *ex vivo* cell culture models to address questions regarding virulence and pathogenesis (307). Taking into account the recently found association of PUUV infection with cardiovascular disease (352) and haemorrhagic complications that may occur during infection, the interaction between PUUV and the coagulation system especially warrants further attention. Since PUUV tends to rapidly lose virulence upon *in vitro* cell passages the use of low passaged isolates is of vital importance (373). Therefore, we have put a lot of emphasis on obtaining low passage PUUV isolates and optimisation of the hemostatic assays under the right biosafety regulations using primary cell cultures.

Based on haemostatic changes seen in several clinical studies, most from Northern Europe, we decided to study specific parts of the coagulation system *in vitro*. We started by studying the effects of PUUV infection on formation of a platelet plug, the major event in primary haemostasis. Binding of platelets by PUUV infected cells could explain thrombocytopenia in acute PUUV patients, since it would result in wasting or loss of platelets adhered to these cells (63;366). Especially if we make notice of the ability of hantaviruses to infect megakaryocytes and thereby lead to a decreased production of platelets, in addition to the loss of platelets adhered to infected cells (374;375). In our model it seems that PUUV infection increases binding of platelets to the surface of HUVEC compared to control cells (Figure 2). Here we assumed increased CD41a expression observed in the first experiments was the result of an increased number of platelets on the HUVEC. Theoretically, increased CD41a detection could also be due to an increased expression of CD41a on platelets, after 30 minutes incubation with infected HUVEC, rather than an actual increase in platelet numbers. While we cannot rule this out based on our experiments, we blocked extrinsic platelet activation by prostaglandin treatment making platelet activation less likely. Furthermore, in line with studies performed with more pathogenic hantaviruses (366), we tested specific binding of platelets to PUUV particles. Judged from results from the platelet pull down experiments (Figure 3) this seemed to be a specific binding between virus and platelets which could be reversed by the addition of PUUV neutralizing antibodies. In these experiments we controlled for aspecific binding of antibodies (isotype control experiments), factors present in the virus culture medium (5 day old VeroE6 medium as a control) and binding of platelet detection antibody directly to PUUV. We expected the increased platelet binding to co-occur with increased VWF production, as a general inflammatory response during infection. However, the observation that VWF concentration does not change during PUUV infection, further suggested a VWF-independent mechanism for platelet binding in HFRS. Results from earlier studies showed an increased VWF concentration in hospitalized PUUV patients (63). One should keep in mind that overall plasma

VWF level in any patient represents the state of the total endothelial cell layer and not only that of infected cells, as is the case in our model. Furthermore we are studying the acute response of endothelial cells in the first 48 hours after infection, a time point at which PUUV patients are generally not considered to be hospitalized and tested. The increase of VWF in all three conditions (control, BPL and PUUV) over time in our HUVEC model could be the result of an increased number of cells or a sign of *in vitro* stress and activation of the endothelial cells. Since it seems highly unlikely the cells still multiply after the formation of a full monolayer, which is present at the time of infection, we believe that also non infected cells show a certain level of activation when in culture.

Gravilovskaya, and colleagues were the first to study the interaction of hantaviruses (Andes and Hantaan) with platelets, and concluded that there was a specific binding of Hantaan and Andes virus particle particles to $\alpha\beta3$ integrins present on both endothelial cells and platelets (366). Interestingly, our experiment showed a trend to increased number of platelets bound to the BPL-inactivated virus treated cultures, suggesting active replication was unnecessary and inactivated virus, bound to the cell surface, might also bind to integrins present on platelets. For the interpretation of our data one should take in mind that we made use of a MOI 3 BPL at $t = 0$ hours and that the BPL inactivated virus will not replicate. Therefore at timepoint $t = 24$ and $t = 48$ the BPL control will most likely be comparable to the MOI 0.5 infection. It could very well be the case that when increasing the MOI for the BPL infection a more comparable result to the MOI 3 infection would be observed.

PUUV infections of HUVEC directly increased the expression of TF on the cell surface and in the cell lysate compared to controls. This resulted in drastic activation of secondary haemostasis in our cell model during PUUV infection. Data from a direct clotting assay on the cell monolayer gives interesting insights in the potential mechanism behind increased thrombin generation seen in acute PUUV patients (134;354). A clear pro-coagulant state, the result of an increased expression of TF on the surface of PUUV infected cells, resulted in enhanced thrombin generation. Increased thrombin generation (decreased thrombin time, overall increase in prothrombin fragments 1+2, antithrombin and protein C) that Laine *et al* observed in acute PUUV patients (354),(134) could very well be the result of direct infection of endothelial cells and concomitant increased production of TF. Whether increase in TF is a general defence response or if the virus actually benefits from TF, as is seen in certain herpesvirus infections (376), remains unknown. However, excess of TF production during infection could lead to increased clotting and eventually consumptive coagulopathy or even DIC, a severe condition that is only seen in a small percentage of PUUV patients (134), but which could be one of the factors contributing to the haemorrhagic complications seen in HFRS. Especially since increased TF expression has been proven to play an important role in the pathogenesis of other viral haemorrhagic fevers like Marburg and Ebola (46;47).

Since alterations in PAI-1 levels are related to renal disturbances comparable to that seen in hantavirus disease (377;378) and functional polymorphisms in PAI-1 were related to more severe disease in acute PUUV patients (379), we also studied PAI-1 and regulators of PAI-1 activity. Infection with PUUV increases PAI-1 production, which would *in vivo* lead to decreased fibrinolysis. The $\alpha\beta3$ integrin receptor plays an important role in PAI-1/vitronectin complex formation (353). Increased $\alpha\beta3$ expression during PUUV infection combined with competitive binding of hantavirus with vitronectin for $\alpha\beta3$ could hypothetically lead to further alterations in PAI-1 half-life and stability. The increased level of vitronectin-PAI-1 complexes in the supernatant of PUUV infected cells further strengthens this hypothesis. Considering that an increase in PAI-1 and vitronectin could result in renal impairment, and even cause a nephritis-like response, pledges for further evaluation of interaction between $\alpha\beta3$ integrin, PAI-1, vitronectin and hantaviruses.

CHAPTER 3.2

A comparative proteomic analysis of Human Umbilical Vein Endothelial Cells after infection with Puumala hantavirus and *Leptospira interrogans*, causative agents of rodent-borne haemorrhagic fevers

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Submitted

Abstract

“Estimates indicate that there are over 1 million cases per year of rodent-borne haemorrhagic fever in humans world-wide. The majority of these cases are caused by infection with pathogenic hantaviruses and Leptospira. Although these are different biological pathogens, Leptospira being spirochetes and hantaviruses being negative stranded RNA viruses, both can cause very similar clinical disease in humans, often characterized by haemorrhage and renal failure. In the present study, a comparative analysis of the proteomic changes in primary isolated, in vitro cultivated human umbilical vein endothelial cells (HUVEC) were characterized after 48 hours of infection with a low passage strain of the Old-World Puumala hantavirus (PUUV) and a virulent isolate of Leptospira interrogans serovar Copenhageni. Both pathogens proved to efficiently infect the endothelial cells and replicate during the 48 hours of incubation without causing any major cytopathic effects. The protein profiles of infected versus non-infected HUVEC and between cells infected with PUUV, Leptospira or in ‘control’ conditions (pathogen free medium) were compared via 2-D differential fluorescent gel electrophoresis (DIGE). Differentially expressed proteins were identified by mass spectrometry. Comparison of infected versus non-infected HUVEC led to the identification of five proteins whose expression was significantly diminished after infection with both pathogens. Comparison of Leptospira-infected versus PUUV-infected HUVEC identified five proteins which were uniquely differentially expressed in PUUV infected HUVEC, suggesting specific roles in replication or (anti-) viral responses in the infected cells. Differentially expressed proteins identified in this study warrant further investigation to understand pathogenic mechanisms of infection, and give a unique insight into the early host response of endothelial cells during infection with PUUV or Leptospira.”

Introduction

Rodent-borne haemorrhagic fevers can be caused by a number of pathogens including pathogenic hantaviruses and *Leptospira* species. Both of these neglected pathogens are able to cause a disease with highly similar clinical features and share many epidemiological characteristics (297). Leptospirosis, is one of the most wide spread zoonotic diseases in the world and the highest incidence is seen in developing countries with a warm and humid climate, but the disease also occurs in industrialized countries (179). It is caused by infection with pathogenic *Leptospira* species, helical shaped bacteria, which are directly or indirectly transmitted by most mammalian species, notably rodents. Clinical symptoms range from a mild febrile disease to a severe triad of hepatic injury, renal failure and haemorrhage, better known as Weil's disease. In the past 20 years, Leptospiral Pulmonary Haemorrhage Syndrome (LPHS) has been increasingly recognized in humans and a number of domestic and wild animal species. LPHS is characterized by severe lung bleedings with a case fatality rate up to 80% (380). Due to the overlapping symptoms, acute leptospirosis can easily be mistaken for the so-called Haemorrhagic Fever with Renal Syndrome (HFRS); the second largest cause of rodent-borne haemorrhagic fever in the world (297). HFRS is the result of infection with a pathogenic hantavirus circulating in Europe or Asia, often referred to as "Old-World" hantaviruses. Pathogenic hantaviruses are transmitted by rodent excreta and transmission occurs after inhalation of virus-containing aerosols (307). Patients with HFRS can present with a wide variety of symptoms (348). However, hallmark symptoms of HFRS are, like in leptospirosis, renal failure potentially accompanied by (severe) bleeding complications (297). HFRS incidence is high in South-East Asia while it is also known to be endemic in large parts of Europe (296).

For both pathogens, the way they cause disease, and especially the way they cause haemorrhage and renal failure, remains largely unknown. Based on *in vitro* and animal and human *in vivo* data, there is a strong suggestion that endothelial cells play a pivotal role in the pathogenesis of both HFRS and leptospirosis. For leptospirosis this assumption is supported by clinical data in severe leptospirosis patients from Indonesia who showed extreme activation of coagulation, which in many cases was followed by (fatal) haemorrhage (381;382). *In vitro* work showed the ability of *Leptospira* to transmigrate through endothelial cell monolayers. Furthermore, outer membrane proteins and killed *Leptospira* cause up-regulation of several markers of endothelial activation like I-CAM and E-Selectin suggesting alterations in the functionality of the vascular endothelium (210;383;384).

In vitro and *in vivo* studies demonstrate that hantaviruses directly infect and replicate in endothelial cells, without causing any cytopathic effects. There is a strong suggestion that the function of endothelial cells is hampered during or due to hantavirus infection (62). For instance, hantavirus infected endothelial cells have a decreased migratory function *in vitro* and

show a VEGF-dependent increase in permeability. Furthermore, platelets tend to adhere to hantavirus infected endothelial cells (360;366).

In order to study the factors involved in the changes in the vascular endothelium in rodent-borne haemorrhagic fever we chose a comprehensive, discovery based, proteomic approach. We hypothesized that both the virus (Puumala hantavirus) and the bacterium (*Leptospira*) directly alter the proteome of endothelial cells and that the specific changes will indicate a mechanism behind the observed endothelial cell dysfunction. Based on the pathogens shared characteristics in clinical manifestations, epidemiology, and asymptomatic reservoir status, we hypothesize that these pathogens might share a similar mode of action on endothelial cells. In other words, the specific changes seen in protein abundance in both infections could very well play an important role in the mechanism behind the endothelial cell dysfunction. Furthermore, we aim to perform the first proteomic analysis of hantavirus-infected endothelial cells compared to uninfected and *Leptospira*-infected controls.

Materials and Methods

Ethics statement

For the use of fresh umbilical veins for primary cell culture written informed consent was obtained and the protocol was reviewed by the Medical Ethics Committee from the Erasmus Medical Center. This committee concluded that the Medical Research Involving Human Subjects Act does not apply on the presented study and that therefore official approval of this study by the Committee was not required. Human umbilical vein endothelial cells (HUVEC) were harvested from fresh umbilical veins from healthy mother and baby after written informed consent for usage in multiple studies in the exotic viruses workgroup of the department of Viroscience, Erasmus Mc, the Netherlands, all for viral haemorrhagic fever pathogenesis studies, including the study presented in this manuscript. Isolated cells were de-identified and therefore could not be traced back to the original donor. For the isolation of Puumala hantavirus Prof. H. Henttonen from at METLA forest research institute, Vantaa, Finland provided lung tissue of *Myodes glareolus* from Konnevesi, Finland, which were collected with permission of the Konnevesi local authorities and performed according to the Cruelty to Animals Act, 1876. The same method has been described in recent PLoS one publications by the group of professor Henttonen (385;386). According to the Finnish Act on the Use of Animals for Experimental Purposes (62/2006) and a further decision by the Finnish Animal Experiment Board (May 16th, 2007), the technique employed to capture rodents, i.e., snaptrapping (the animal capture technique, i.e., using traps that instantly kill the animal) that was used is not considered an animal experiment and therefore requires no animal ethics license from the Finnish Animal Experiment Board. The species captured for this study, *Myodes glareolus*, neither is protected nor included in the Red List of Finnish Species. Animal trapping took place

on private and Finnish national forest by permit (1013/204/2002). Landowners were consulted and the trapping was allowed before the study was conducted.

The isolation and ethical approval for the *Leptospira interrogans* serovar Copenhageni (RJ16441) has been described earlier in (387) for this study we used the first passage isolate of the leptospire and no longer the blood or any samples of the patient was required. For the housing and passages of the RJ16441 J. Nally and S.S. Schuller had ethical approval from the ethics committee of the University College Dublin, Dublin, Ireland.

Cells

Human umbilical vein endothelial cells (HUVEC) were harvested from fresh umbilical veins from healthy mother and baby . Isolated cells were de-identified and therefore could not be traced back to the original donor. Umbilical cords were stored in sterile 500 ml PBS + gentamycin (50 µg/ml) (Leo Pharmaceutical Products, Denmark). The veins were rinsed with PBS containing 50 U/ml heparin (Leo Pharmaceutical Products). Subsequently, cells were detached with 0.1% collagenase solution (C6885, Sigma Aldrich, USA). Cell suspension was collected in a sterile 50 ml tube followed by centrifugation (2 x 5 minutes; 300 x g). The cell pellet was re-suspended in HUVEC medium (human endothelial-SFM medium, Invitrogen, USA) containing 10% human serum (Lonza, the Netherlands), 20% filtrated Fetal Bovine Serum (FBS); penicillin/streptomycin 100 U/ml, 20 ng/ml fibroblast growth factor (Peprotech, USA) and 10 ng/ml of endothelial cell growth factor (Peprotech). Cell suspensions were cultured in flasks pre-coated with 20 µg/ml of fibronectin (Roche, the Netherlands). Passage two cells from one specific donor were used for this study. The identity of the endothelial cells was confirmed by flow cytometry using *Ulex europeus* lectin (EY laboratories, USA), anti-CD31 antibody (Sigma Aldrich, USA) and Von Willebrand Factor staining (Dako, the Netherlands).

Virus and Bacteria

Puumala (PUUV) hantavirus was isolated from lungs of *Myodes glareolus*. Virus was isolated by an in-house developed protocol (Chapter 3.1). Briefly, lungs were homogenized (10% w/v) and added onto veroE6 cells and incubated for 60 minutes (37°C; 5% CO₂). Supernatant was discarded and cells were washed three times and incubated with fresh veroE6 medium (Dulbecco's Modified Eagle Medium containing 10% FBS, 100 U/ml penicillin-streptomycin solution, 1% HEPES buffer and 1% sodium bicarbonate (all from Gibco, Life Sciences, USA) for an additional five days. Virus stocks of up to passage four were created. The virus titre was determined using immune peroxidase reaction (IPOX), and TCID₅₀ was calculated using the Karber formula (367). A low passage isolate of *Leptospira interrogans* serovar Copenhageni (RJ16441) was used, originally isolated from blood cultures of a clinically infected human patient with LPHS as described in (387). The organism was maintained in Ellinghausen-

McCullough-Johnson-Harris (EMJH) semisolid medium (EMJH medium containing 0.2% noble agar and 200 µg/ml 5-fluorouracil) or liquid culture medium (EMJH medium with 6% rabbit serum) at 30°C under aerobic conditions and regularly passaged through guinea pigs to preserve its virulence (for which ethical approval was given by the University College Dublin). *Leptospira* were harvested at the log phase of their growth ($1-2 \times 10^8$ /ml) and washed 2 times in RPMI medium before being re-suspended in HUVEC medium.

Infection

In a 24 well plate, 2.4×10^5 endothelial cells were incubated with a multiplicity of infection (MOI) of 3 either with PUUV, *Leptospira*, or normal HUVEC medium (without antibiotics, with 20% FBS and no human plasma) as a control. After 48 hours of incubation, HUVEC were washed three times with 10 mM Tris 1 mM EDTA buffer (pH 8). Proteins were solubilised by adding 100 µl solubilisation buffer (7 M urea, 2 M thiourea, 1% Amidosulfobetaine-14). Samples were stored at -80°C until further analysis.

Infection kinetics

Infection of endothelial cells was assessed by immune peroxidase staining (IPOX). Briefly, supernatant was discarded from a full monolayer of 24 well plate seeded HUVEC and cells were washed three times with phosphate buffered saline (PBS). Because it was unclear whether the leptospires would remain extracellular or migrate intracellular, a two-way strategy for antigen detection was used. At the given time point, cells were treated either with permeabilization (100% -20°C methanol fixation) or without permeabilization (4% formalin fixation). Cells were fixed and incubated at -20°C for 30 minutes. Methanol was discarded and cells were incubated for 60 minutes at 37 °C with 100 µl 0.05% H₂O₂ in PBS, to block endogenous peroxidases. Cells were washed three times and incubated (60 minutes) either with 500µl rabbit polyclonal anti-*Leptospira interrogans* serovar Copenhageni (1:500, KIT Biomedical Research, The Netherlands) or with 500µl rabbit anti-Puumala virus nucleoprotein (1:500, Bei Resources, USA) antibodies. Cells were washed (PBS 0.05% tween) followed by incubation with HRP-labelled goat anti-rabbit IgG conjugate (1:500). Antibodies and conjugate were diluted in dilution buffer (PBS 0.5% BSA, 2% NaCl and 1% normal goat serum). Colour development was achieved by addition of 3-amino-9-ethylcarbazole (AEC) substrate (AEC dissolved in dimethylformamide buffered with acetate buffer pH 5). The percentage of infected cells was determined by manual counting.

Viral replication was also determined by RNA copy number quantification. The viral RNA quantity was measured with a real-time qRT-PCR assay (TaqMan®Fast Virus 1-Step Master Mix, Invitrogen, Life Sciences) using Applied Biosystems® 7500 Real-Time PCR system. The RNA copy number in each sample was calculated from a standard curve generated by *in vitro* transcribed PUUV RNA standard. RNA run-off transcripts were generated using a segment amplified with pan-hantavirus degenerative PCR primers as described in (370) . PCR products were separated

on 1% agarose gel and bands of correct size were collected for DNA gel extraction using MinElute Gel Extraction Kit Protocol (Qiagen, United States). DNA fragments were cloned into the pCR4 vector using the TOPO® TA Cloning KIT (Life Technologies) and One Shot® TOP10 chemically competent E.coli were transformed with the recombinant vector (QIAGEN, California). At least five colonies were collected for further analyses. Plasmid DNA was purified using MinElute DNA purification kit (QIAGEN). Plasmid DNA was linearized by restriction digestion (NotI for negative strand RNA and PstI for positive strand RNA). Run-off transcripts were synthesized using T3 RNA polymerase for negative strand and T7 RNA polymerase for positive strand (MEGAscript® T3 and T7 transcription kits, Life technologies), followed by DNase treatment (Ambion® TURBO DNA-free™ Life Technologies), according to manufacturer's manual. The amount of RNA in the stock was determined using nanodrop. Copy numbers in the standards were calculated using RNA concentration and sequence length with a calculator (<http://endmemo.com/bio/dnacopynum.php>).

Leptospira were quantified using a real-time TaqMan® based quantitative PCR based on the *lipI32* gene for the quantitation of pathogenic *Leptospira* species based on the protocol described in (388). The DNA copy number per sample was calculated from a standard curve generated by a serial dilution of cultured leptospires counted by darkfield microscopy with the use of a Helber counting chamber.

1 Dimensional (1-D) Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide gels with a discontinuous buffer system. For 1-D gel electrophoresis, resolving gels were cast using 12% polyacrylamide, 1.5 M Tris-buffer at pH 8.8, 0.1% SDS, ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and then overlaid with a stacking gel to concentrate the protein prior to separation (4% polyacrylamide, 0.5 M Tris-buffer pH 6.8, distilled water, 0.1% SDS, APS, TEMED). Samples from hantavirus, leptospirosis or mock control infections were defrosted and protein concentration was quantified using RC/DC (Biorad, LifeScience research). 5 µg of protein was mixed with an equal volume of 2 x sample loading buffer (1 M Tris/HCl pH 6.8, 2% beta-mercaptoethanol, 4% SDS, 20% glycerol, 0.2% bromophenol blue) and boiled for 10 min in order to achieve SDS-binding and denaturation. Gels were placed into a Mini-Protean II cell (Bio-Rad) and the chamber then filled with SDS electrophoresis buffer (25mM Tris base, 0.192 mM glycine, 0,1% SDS, pH 8.3). Samples were loaded into the precast loading bays of the stacking gels. A molecular marker (MagicMark, Invitrogen) was loaded into the first lane of each gel. Electrophoresis was then performed for about 70 min at 160 V until the tracking dye contained in the sample buffer ran off the lower end of the gels.

2 Dimensional (2-D) Gel Electrophoresis

For 2-D gel electrophoresis, proteins were first separated according to their isoelectric point using 7 cm immobilised pH gradient (IPG) strips with a pH range of 4-7 and 3-10 (GE Healthcare). Strips were rehydrated overnight in rehydration solution containing 30mM DTT, 0.5% IPG buffer, bromophenol blue, protein sample and solubilisation buffer (7 M urea, 2 M thiourea, 1% ASB-14) to a final volume of 126 μ l or 450 μ l per 7 cm strip respectively. Isoelectric focusing was performed using an Ettan IPGphor IEF System (GE Healthcare) using the settings detailed in Table 2. After isoelectric focusing, strips were either processed immediately or frozen at -80°C until further use. For second dimension separation, strips were defrosted and transferred into SDS equilibration buffer (6M urea, 75mM Tris-HCl, pH 8.8, 29,3% glycerol, 2% SDS and 0.002% bromophenol blue) with 1% DTT for 10 min. followed by a second equilibration buffer containing 2.5% iodoacetamide for 10 min. The strips were rinsed with electrophoresis running buffer and overlaid to 12% polyacrylamide gels and sealed with agarose gel with bromophenol blue (tracking dye). Gels were run at 160 V for 70 min in a Mini-Protean II cell (Bio-Rad).

2-D DIGE

To comparatively analyze protein abundance in HUVEC, cells incubated either with PUUV, leptospires or a control infection, were labelled using Cy3 and Cy5 dyes for 2D-DIGE analysis. The design and flow of this experiment is summarized in Figure 1. Dyes were received frozen and kept at -20°C until use (Interchim, France). The dyes were reconstituted in dimethylformamide (DMF) to 1mM stock solutions. The stock solutions were then further diluted with DMF to 400 pmol/ μ l working solutions. The Cy2 label was used for the internal control which was used Foron each gel. The internal control was made from, 50 μ g of protein of an internal standard, consisting of a pool of equal protein amounts from all samples included in the experiment, was used and labelled with Cy2. While 50 μ g of protein from PUUV, leptospirosis or control infected endothelial cells were labelled with 400 pmol (1 μ l) Cy3 or Cy5. The samples were left to incubate on ice for 30 min. The labelling was then quenched by addition of 1 μ l of 10mM lysine per μ l of CyDye. After labelling the samples were separated in first and second dimension as described under 2D-SDS PAGE electrophoresis except using 24 cm pH 4-7 IEF strips. During the entire experiment the samples were protected from light in order to prevent degradation of the CyDye labels. After protein separation, gels were scanned using the Typhoon fluorescence gel scanner (GE Healthcare) with the following wave lengths: 520 nm for Cy2, 580 nm for Cy3 and 670 nm for Cy5. The gels were pre-scanned at a pixel size of 500 μ m and the photomultiplier tube (PMT) voltage adjusted in order to ensure that the spot intensities for each CyDye were within a range of 60000-80000U. For data analysis gels were scanned at a pixel size of 100 μ m.

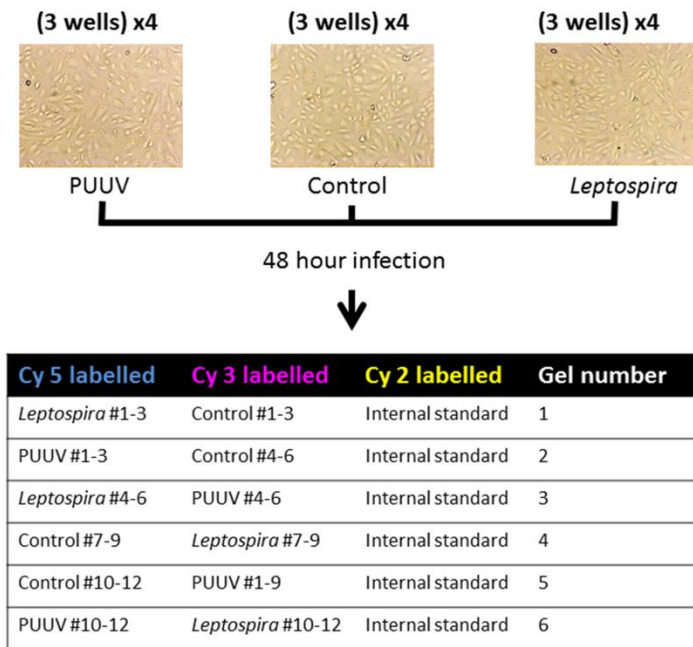


Figure 1. DIGE experimental design for the analysis of differentially expressed proteins between non infected, Puumala hantavirus infected and *Leptospira interrogans* Copenhageni infected Human Umbilical Vein Endothelial cells 48 hours post infection.

Statistical and spot analysis

All statistical analyses for infection kinetic experiments were performed using the software GraphPad Prism 4.01 for Windows. The data were expressed as mean or median with or without standard deviation or 95% confidence interval. Differences with $p \leq 0.05$ were considered statistically significant.

Differences in spot volumes were analyzed by use of the ProgenesisSameSpots® software (<http://www.nonlinear.com>) following the producer's instructions. Gels were aligned and the spot volumes of the individual samples normalized against the internal standard. Statistical criteria for significant differences in spot volumes were: $p \leq 0.05$, $q \leq 0.2$ and power ≥ 0.9 .

Protein digestion and Identification

The differentially expressed protein spots were excised from 2-D DIGE gels and manually digested with trypsin. After digestion, the peptides were solubilized in 2 μ l (acetonitrile 50%, TFA 0.1%) and 0.7 μ l of the peptide mixture spotted on a MALDI target plate. A total of 0.7 μ l of matrix solution (alpha cyano-4-hydroxycinnamic acid in 50% ACN/acetonitrile/ 0.1%

trifluoroacetic acid (TFA) was added. All MS and MS/MS analyses were performed using a 5800 MALDI TOF/TOF (Applied Biosystems, Foster City, CA, USA) internally calibrated with the known masses of trypsin autocleavage products in MS and externally with fragments from Glu-fibrinopeptide in MS/MS. For each sample, one MS spectrum was acquired and the 10 most intense precursors were subsequently selected for MS/MS analysis. An ABSciex ProteinPilot platform was used for database searches on an in-house MASCOT server (version 2.3, Matrix Science, matrixscience.com, London, UK). Peaks with a signal to noise ratio of more than 10 for MS-analysis and more than 4 for MS/MS analysis were included in the peak list. Combined MS and 10 MS/MS spectra from each spot were used to perform a search against a Swissprot database (downloaded on the 21st January 2013) with the taxonomy Homo sapiens database (20307 sequences) and subsequently against all entries (542782 sequences). A mass window of 100 ppm for the precursor and 0.5 Da for the fragments was tolerated. During the database searches, the following parameters were defined: two missed cleavages, fixed carbamidomethylation of cysteine, variable oxidation of methionine and tryptophan, tryptophan to kynurenine or double oxidation to N-formylkynurenine. Proteins were considered as being identified when two peptides matched with a score above 40, the peptide threshold score or when one high-scoring peptide together with the MS-data resulted in a protein expect value <0.001 . All identifications were manually validated, as previously described (389). During the acquisition of data, an effort was made to explain as many as possible of the peaks observed in the MS spectra, resulting in an increase in the sequence coverage for the reported identifications, the identification of multiple proteins in an important number of spots. The biological meaning of identified semi-tryptic peptides (peptides partially resulting from non-tryptic cleavages), was searched using SignalP or MitoProt (expasy.org/tools) for the prediction of signal- and transit-peptides respectively.

Functional pathway analysis of identified proteins

Protein identifications were imported into IPA[®] and mapped to items in the Ingenuity[®] knowledge base to identify known regulatory pathways. Core analysis was performed for proteins with significant changes in abundance between infected HUVEC versus non-infected HUVEC followed by the hantavirus specific analysis (PUUV infected HUVEC versus non-infected + *Leptospira* infected cells).

Results

Infection of HUVEC

Given our hypothesis that rodent-borne haemorrhagic fevers effect endothelial cells in a similar manner, infected HUVEC were examined at 48 hours post-infection with a MOI 3 of either PUUV or *Leptospira* in order to identify those initial protein changes in infected cells prior to any cytopathological changes. This was based on previous work by Kraus and colleagues who observed a potent interferon response in endothelial cells during hantavirus infection that plateaued at 48 hours post-infection (390). Similarly, evaluation of low and moderate MOI's using low passage PUUV on hemostatic parameters in endothelial cells have determined significant changes by 48 hours in the absence of any apparent cytopathological changes (Goeijenbier *et al.*, manuscript in review).

The morphology of PUUV infected HUVEC did not differ from non-infected controls over the time course of infection (Figure 2A & B) and no cytopathological effects were observed. Infection was visualized by IPOX, which confirmed the intracellular presence of PUUV nucleoprotein in over 90% of cells (Figure 2D). The 2log increase in viral copy numbers detected in the supernatant of infected HUVEC (Figure 2E) and the 3log increase in viral copy numbers measured in the HUVEC cell lysate confirmed efficient replication of PUUV in this primary culture HUVEC model.

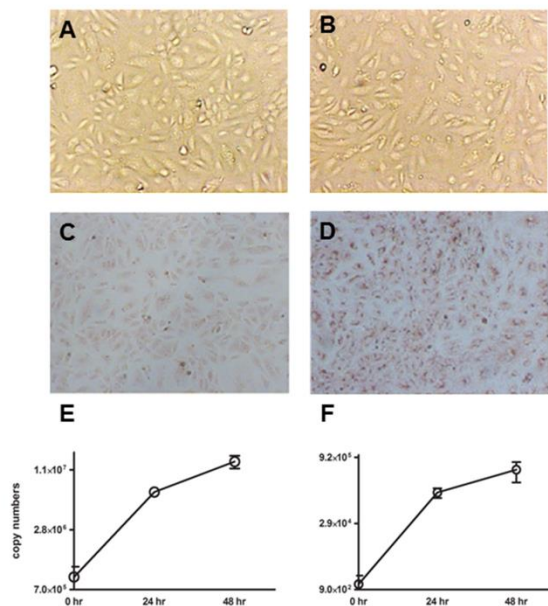


Figure 2. Infection of HUVEC with Puumala hantavirus.

The morphology of non-infected Human Umbilical Vein Endothelial cells (HUVEC) (2A) and HUVEC infected with a Puumala hantavirus multiplicity of infection of 3 (2B) is shown in the upper two panels. The morphology of the HUVEC did not seem to differ between the two conditions and the cells did not show any obvious cytopathology effects over the time course of infection. Red positive staining in the Puumala hantavirus infected HUVEC 48 hours post infection (2D) shows the presence of Puumala hantavirus nucleoprotein in almost 100% of the cells, confirming infection. The 2log increase in viral copy

numbers seen in the supernatant of infected HUVEC (2E) and the 3log increase in viral copynumbers measured in the HUVEC cell lysate (2F) confirms efficient and effective replication of the Puumala hantavirus in the HUVEC model.

The morphology of *Leptospira* infected HUVEC did not differ from non-infected controls over the time course of infection (Figure 3A & B) and no cytopathological effects were observed. Immunohistochemistry and PCR confirmed the infection of HUVEC with *Leptospira*. In order to determine if the *Leptospira* remained extracellular or penetrated the endothelial cells, to survive intracellular, immunohistochemistry was performed with and without permeabilization of the cells (Figure 3D & 3E). Extracellular *Leptospira* were detected in the absence of permeabilization (Figure 3D) whilst permeabilization allowed detection of multiple intracellular leptospires (Figure 3E). Quantitative real time PCR showed that the number of leptospires did not change in the cell supernatant (Figure 3F) whilst it almost doubled in the cell lysate (Figure 3G) ($p = 0.0022$ Mann Whitney-U test). Results indicate a generation time of approximately 24 hours which is typical of pathogenic isolates of *Leptospira* in culture (391) and confirms replication of *L. interrogans* in our HUVEC model.

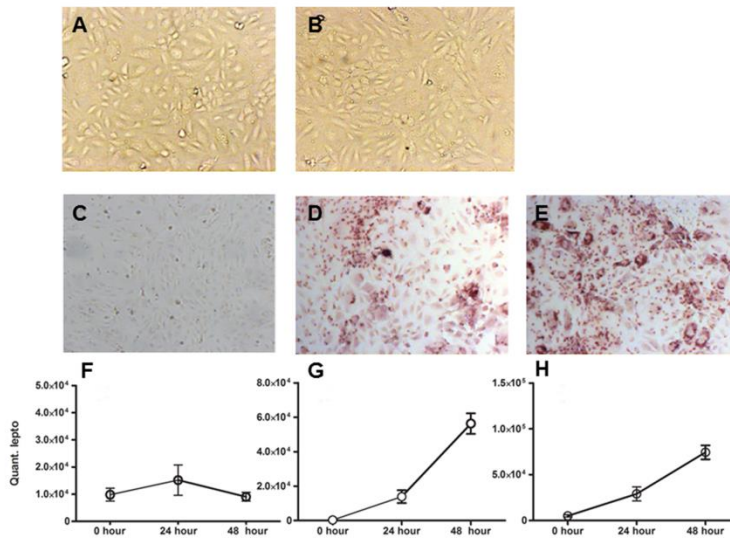


Figure 3. Incubation of HUVEC with *Leptospira interrogans* Copenhageni.

The morphology of non-infected Human Umbilical Vein Endothelial cells (HUVEC) (3A) and HUVEC 48 hours incubated with a *Leptospira interrogans* multiplicity of infection of 3 (3B) is shown in the upper two panels. The morphology of the HUVEC does not seem to differ

between the two conditions and the cells do not show any obvious cytopathology effects over the time course of infection. In order to determine if *L. interrogans* remained extracellular or had penetrated HUVEC cells to survive intracellular, immunohistochemistry was performed. Compared to the control cells (3C), HUVEC incubated with *Leptospira* stained positive for the presence of the bacteria 48 hours after incubation (3 D&E). We performed a staining with a rabbit polyclonal anti- *L. interrogans* antibody without permeabilization of the cells

(paraformaldehyde fixation; 3D) to show the extracellular presence of *Leptospira* and a staining with permeabilization of the cells (methanol fixation; 3E) which showed clear intracellular presence of *Leptospira*. Survival and replication was further confirmed by a quantitative real time PCR where the amount of *Leptospira* could be calculated from a standard curve. The number of leptospire did not change in the supernatant (3F) while it almost doubled in the cell lysate (3G) which remained a significant increase over time when the results of the supernatant and cell lysate were added up for each specific well (3H).

Altered protein abundance in HUVEC infected with PUUV or *Leptospira*

No significant differences in protein abundance were detected between the cell lysates from PUUV, *Leptospira*, or control infected endothelial cells when compared by 1D-PAGE (Figure 4). Despite absent cytopathology in cells infected either with PUUV or *Leptospira*, functional changes and differences in cytokine production have previously been observed (62). However, evaluation by 2-D PAGE protein separation showed differences in the spot pattern and spot intensities (Figure 5). For example, three protein spots that are diminished after infection with PUUV and *Leptospira* were observed in the 3-10 pH range (Figure 5). Due to increased spot resolution, more differences in spot patterns are detectable by 2D gel electrophoresis in the 4-7 pH range. Given the apparent differences in the proteome between PUUV- and *Leptospira*-infected HUVEC compared to the control condition in the 4-7 pH range, a DIGE experiment was performed.

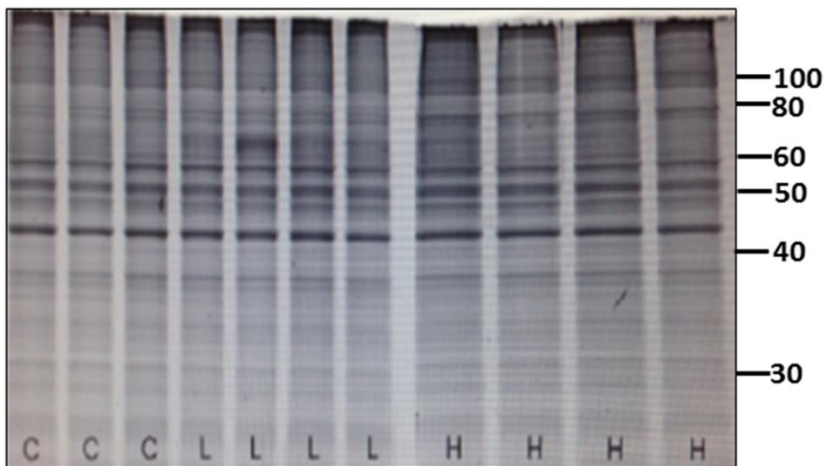


Figure 4. 1D-SDS-PAGE of HUVEC lysate 48 hours after infection with PUUV, *Leptospira* *copenhageni* or a mock control.

1D SDS PAGE gel of cell lysates of HUVEC's infected with a control infection (C), *Leptospira* *copenhageni* (L) or Puumala hantavirus (H). 10 μ g protein from the three different conditions were loaded per lane and separated via 1-D SDS-PAGE. Gels were fixated with 10% methanol, 7% glacial acetic acid followed by Sypro Ruby staining.

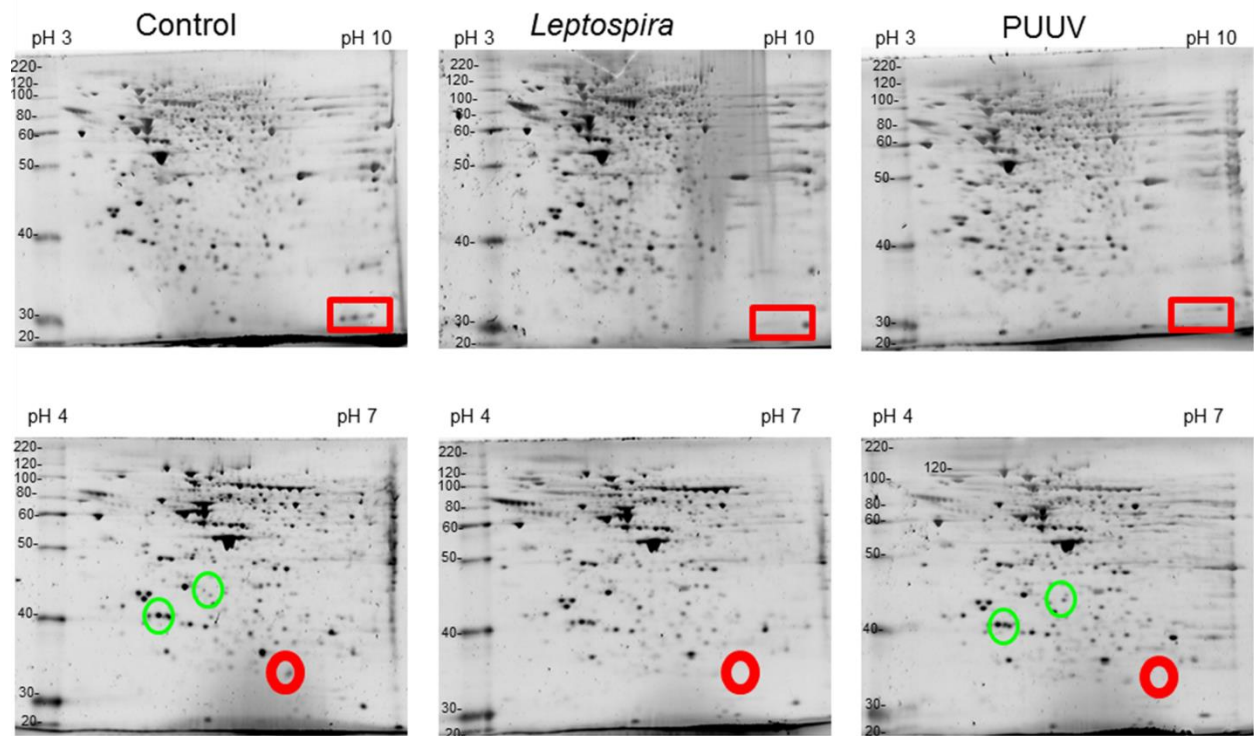


Figure 5. 2-D-SDS-PAGE electrophoresis of HUVEC lysate 48 hours after infection with a medium control, *Leptospira copenhageni* or Puumala hantavirus (PUUV). *SyproRuby* stained proteins from *in vitro* cultured endothelial cells infected with *Leptospira*, Puumala hantavirus or a control infection separated in the first dimension with a PH range from 3-10 and 4-7. Good protein separation is achieved with the majority of the proteins having an apparent molecular weights between 25 and 220 kDa. The red square shows three clear proteins present in the pH 3-10 separation in the control condition which are absent in both *Leptospira* and PUUV infected HUVEC. Red squares in the pH4-7 separation show a clear example of a protein present in the control condition and absent or severely reduced in abundance in both infections while the green circle show two examples of proteins with higher expression in the PUUV infected HUVEC after 48 hours of incubation.

In order to account for biological and technical variation, samples from 3 wells (each containing 2.4×10^5 HUVEC) were pooled to provide one sample for Cydye labelling resulting in a total of 12 (3 x 4) biological replicates for each of the three conditions that were compared over six gels (Figure 1).

In total, 1228 proteins were aligned between the three conditions over 6 gels (Figure 6). Comparison of the proteome of infected HUVEC with non-infected controls, identified 97 (7.9%) of 1228 protein spots with significant differences in the mean normalized spot volume ($p < 0.05$, $q < 0.2$ and power > 0.9) (Figure 8 & Table S1). Eighteen of the 97 proteins were present in greater abundance (1.1-1.6 fold change; Table S1) compared to 79 which were present with a lower abundance (1.8-13 fold range) (Figure 6 & Table S 1). Standardized normalized expression profiles showed proteins which were decreased (Figure 8A) or increased (Figure 8B) in infected HUVEC compared to non-infected controls.

When PUUV infected HUVEC were compared to non-infected controls, there were 36 proteins (2.9%) with an altered abundance of which 25 proteins were increased ($p \leq 0.05$, $q \leq 0.2$ and power ≥ 0.9 , 1.3-21 fold range) compared to 11 proteins that were decreased ($p \leq 0.05$, $q \leq 0.2$ and power ≥ 0.9 , 4.6-16 fold range) (Figure 6B and Table S1). Subsequently, the design of this comparative proteomic approach facilitated comparison of the proteome of PUUV-infected HUVEC with *Leptospira*-infected HUVEC (Figure 7B). In this way, differentially expressed proteins specific for PUUV infected HUVEC, and not those regulated as a result of a more general inflammatory response, could be identified. Comparison of the proteome of PUUV-infected HUVEC with *Leptospira* infected HUVEC identified that 15 of the 25 proteins that were increased in abundance were specific to PUUV infected HUVEC whilst 10 were increased in both PUUV-infected and *Leptospira* infected HUVEC ($p \leq 0.05$, $q \leq 0.2$ and power ≥ 0.9 , 1.6-15 fold range) (Figure 7B). All proteins with decreased abundance in PUUV infected HUVEC were also decreased in the proteome of HUVEC infected with *Leptospira* (Figure 7B).

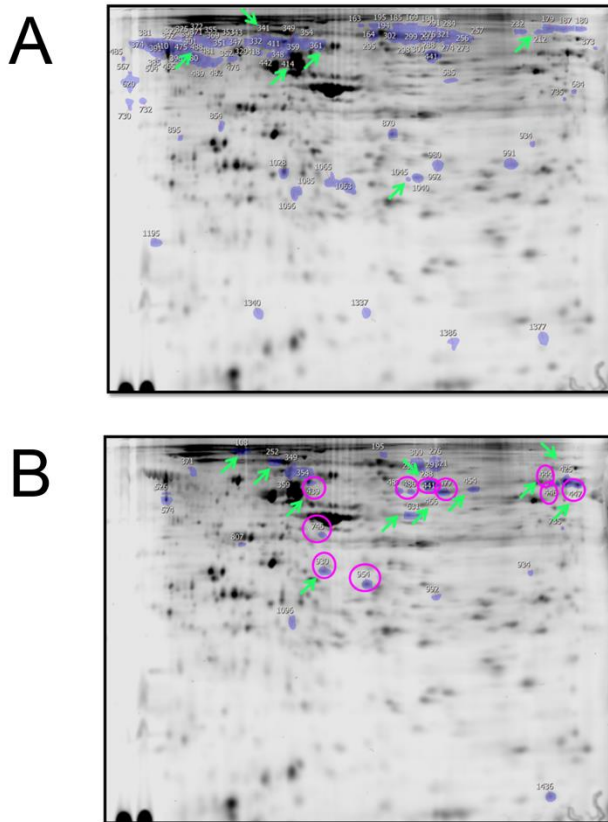


Figure 6. 2D-map of HUVEC. Differentially expressed (DE) proteins identified between infected and non-infected HUVEC (4A) and Puumala hantavirus HUVEC compared to non-infected HUVEC (4B). Proteins identified by mass spectrometry are highlighted by a green arrow. In 4B proteins highlighted by a purple circle are those proteins that also showed a significant altered protein abundance when compar Puumala hantavirus infected HUVEC with *Leptospira interrogans* infected HUVEC. Spot number corresponds to the numbers as listed for the identified proteins in Table 1 and for all DE proteins in Table S1.

up = total number of protein increased in abundance ($p \leq 0.05$, $q \leq 0.2$, Power ≥ 0.9)
 # down = total number of protein decreased in abundance ($p \leq 0.05$, $q \leq 0.2$, Power ≥ 0.9)

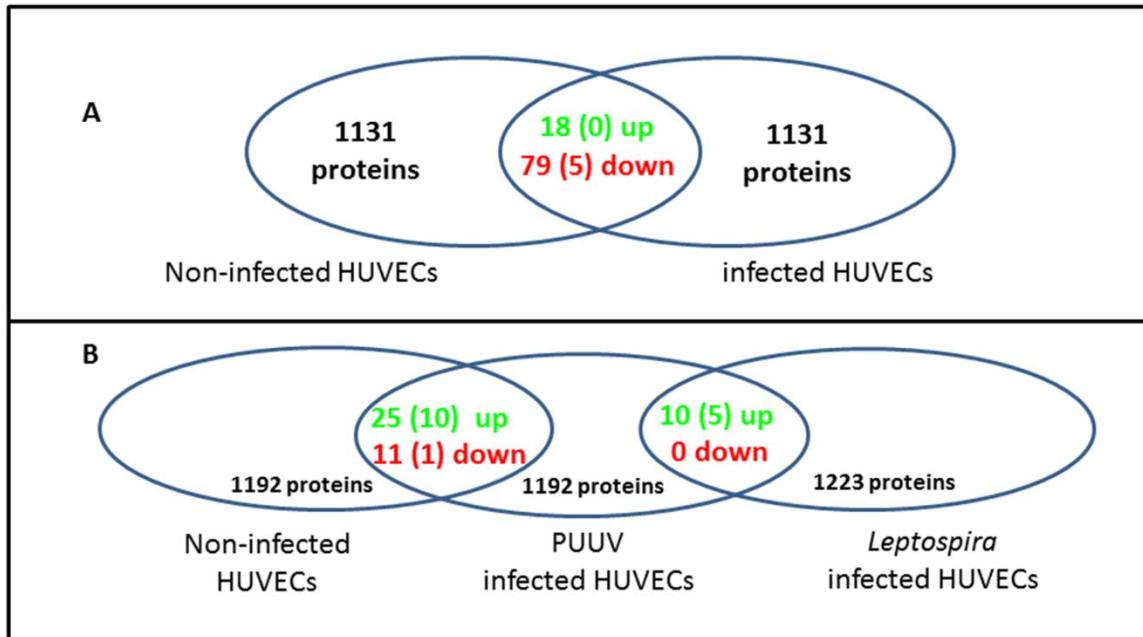


Figure 7. Venn-Diagram of the differentially expressed (DE) proteins between control, Puumala hantavirus and *Leptospira interrogans* infected HUVEC. We used the data on protein abundance from the 2D-DIGE analysis for two different analysis. At first we compared the proteome of infected HUVEC (Puumala hantavirus and *Leptospira*) to non-infected HUVEC (Panel A). This resulted in 18 up regulated proteins and 79 down regulated proteins after p , q and power analysis. The number between brackets represent the number of proteins identified by mass spectrometry. Subsequently we performed the first analysis of the proteome of hantavirus infected endothelial cells to non-infected cells which resulted in 25 up-regulated and 11 down-regulated proteins of which 10 upregulated proteins also met the p , q and power criteria when compared to the *Leptospira* infected cells.

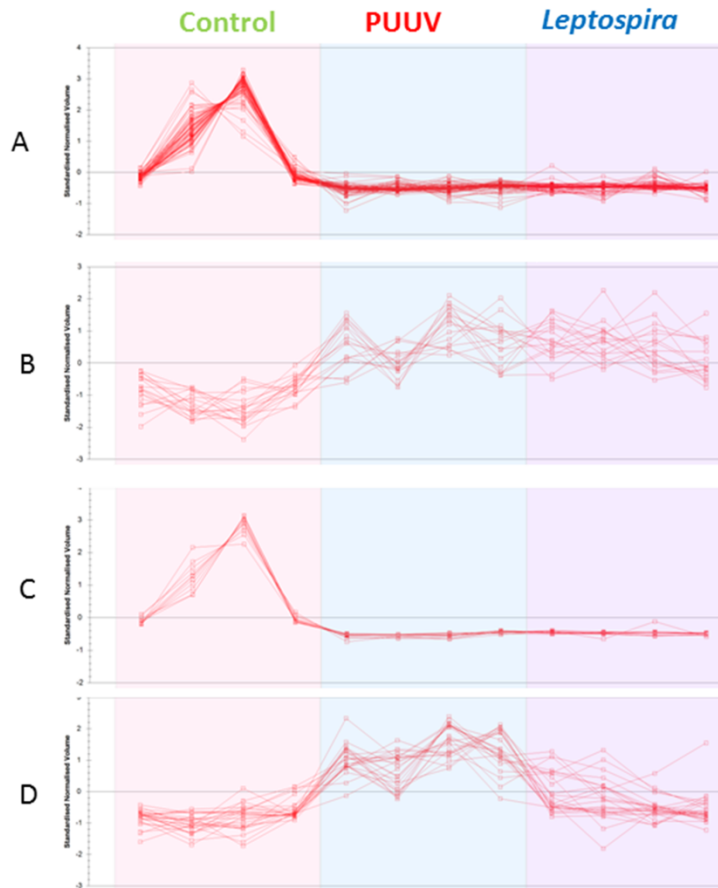


Figure 6. Standardized normalized volume for all spots that met statistical criteria. The standardized normalized volume for all spots with a different protein abundance that met the statistical criteria of $p \leq 0.05$, $q \leq 0.2$ and $\text{Power} \geq 0.9$. Panel A shows the down regulated proteins when comparing infected versus non-infected HUVEC while panel B shows the up regulated proteins from this analysis. Standardized expression volume in the analysis between Puumala hantavirus infected versus non-infected and *Leptospira* infected endothelial cells are shown in panel C (down regulated) and panel D (up regulated).

Identification of differentially expressed proteins

Of the five proteins identified by mass spectrometry in the analysis between infected and non-infected HUVEC, all were decreased in abundance and most are predicted to be cytoplasmic. The identified proteins consisted of cytoskeleton proteins, a transporter and an enzyme (Figure 5 & Table 1). The expression of vimentin, a type III integral filament essential for cell shape and stabilizing cytoskeletal interaction, was consistently identified in lower amounts in infected HUVEC compared to the uninfected controls. Vimentin, identified in spot 488, showed a decreased fold-change of 5.1 with a matching p-value of 0.001. Another protein that plays an important role in actin formation, heat shock protein beta-1, was identified from spot 1045, and was 3.1 times down-regulated in infected cells. Furthermore, a 60 kDa heat shock

protein (spot 361), which plays a role in mitochondrial protein folding and assembly, showed a 7.1 fold down-regulation in the infected cells ($p = 0.0008$). The remaining proteins whose abundance was decreased after infection comprised albumin (8.8 fold change; $p = 0.0003$) and keratin, type II cytoskeletal 1. Two isoforms of the cytoskeletal keratin were identified (protein spot 212 & 414) and both isoforms showed >3 fold decrease after infection. In endothelial cells, cytoskeletal keratin type II, regulated by the KRT1 gene, is membrane bound and has been reported to participate in complement activation, fibrinolysis, angiogenesis and the response to oxidative stress (392).

Of the 12 proteins identified by mass spectrometry in the comparison between PUUV-infected and non-infected cells (Figure 5B & Figure 6B), 10 proteins were increased in abundance whilst one was decreased (Table 1). The 5 proteins which were increased in abundance in PUUV-infected cells compared to non-infected cells and compared to *Leptospira* infected cells and identified by mass spectrometry include 2 peptidases, 1 enzyme, 1 transporter, and 1 pre-mRNA binding protein. Four of these five proteins have been reported in the literature to play a role during viral infection (Table 1). Protein disulfide isomerase (2.2 fold change; $p = 0.002$) is the only protein in this group that has been associated with infection with another causative agent of viral haemorrhagic fever, as this protein is up regulated during dengue virus infection. Leucine aminopeptidase 3 was identified from spot 447. This multifunctional amino peptidase showed a 3-fold increase in the PUUV-infected HUVEC with excellent reproducibility over the biological replicates (Figure S3). Furthermore, N-ethylmaleimide-sensitive factor attachment protein alpha (α SNAP), encoded by the NAPA gene, was 1.6 fold upregulated after hantavirus infection. The α SNAP protein is important in intracellular membrane fusion and vesicular trafficking and specifically in the transport of Weibel-Palade bodies in endothelial cells (393). Furthermore, when comparing the proteome of PUUV infected endothelial cells and *Leptospira* infected endothelial cells, pre-mRNA processing factor 19 showed a 2.3 fold increased protein abundance.

Proteins that showed a significant increased protein abundance after PUUV infection compared to non-infected cells but no significant increase when compared to HUVEC infected with *Leptospira* included: "Heat shock protein 90kDa beta member 1, Tryptophan-tRNA ligase, Selenide water dikinase 1, Heat shock 70kDa protein 5 and Aldehyde dehydrogenase 1". The replicate results from 2D-DIGE analysis of Heat shock 70kDa protein 5 (3 fold up-regulated, $p = 1.8e-05$) is shown in Figure 9. This protein is known to play a role in the assembly of multimeric proteins and was proven to be involved in Ebola and Marburg infection *in vitro* (Table 1). Compared to *Leptospira*-infected HUVEC, protein abundance of this protein was 2.2 fold increased after PUUV infection, however due to a q-value above the threshold it did not reach statistical significance (Table S1). The only protein with a decreased expression after hantavirus infection that could be identified by mass spectrometry was albumin, a known negative acute

phase protein. No leptospiral or viral proteins were identified by mass spectrometry. In multiple spots, proteins derived from fetal bovine serum (FBS) were identified (Dataset S5). The amount of FBS added to cell culture (20%) was consistent in all three conditions and therefore FBS derived proteins were not considered to be not differentially expressed and excluded from data analysis. In spot 439, four proteins were identified (Table 1), of which all four theoretically could have a potential role in the early host response to PUUV infected endothelial cells. However, additional analysis is required to identify the protein(s) responsible for the increased abundance measured in the spot.

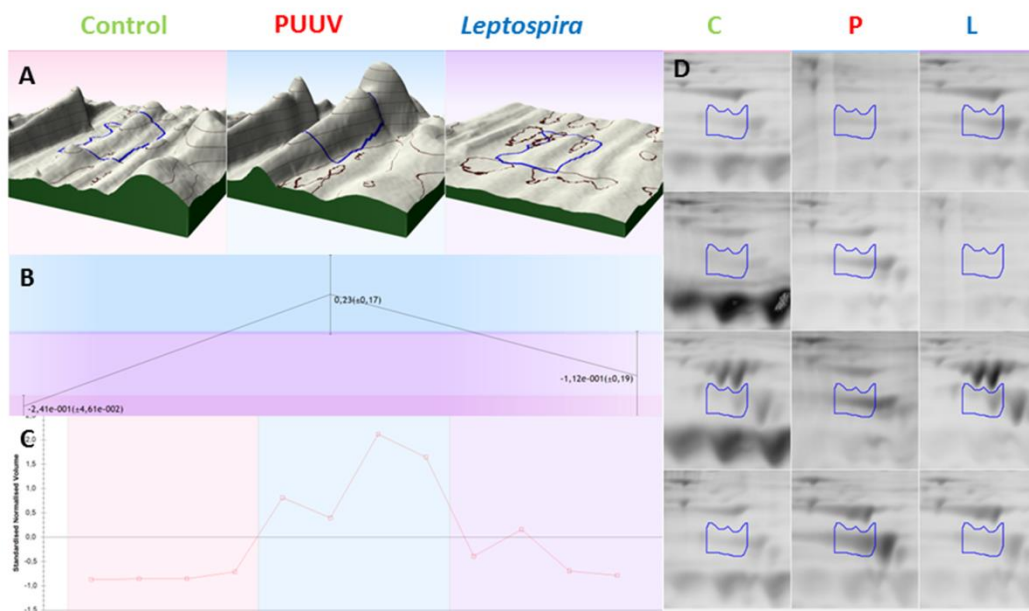


Figure 9. Example of a differential expressed protein. Protein with spot number 252: up regulated 3 fold in Puumala hantavirus infected HUVEC $p = 0,000188$; $q = 0,04$ and power = >0.9995 ; Identified by MALDI TOF as heat shock 70kDa protein

ID & spot	fold	UP/Down	P	Power	q	Score	Protein & Location	Swiss prot	Literature summary	Ref.
Infected versus Non-infected HUVEC										
VIM 488	5.1	Down	0.001	0.98	0.03	97 (+64)	Vimentin (cytoplasm)	Class-III intermediate filaments	Virology: receptor, essential for replication and virus survival. Endothelial cell: migration, adhesion and sprouting.	(394)
HSPB1 1045	2.9	Down	0.002	0.97	0.03	161	Heat shock 27kDa protein 1 (cytoplasm)	Involved in stress resistance and actin organization	Virology: essential for Influenza A replication.	(395)
HSPD1 361	7.1	Down	0.0008	0.99	0.02	90 (+31)	Heat shock60kDa protein 1 (cytoplasm)	Enzyme; Implicated in mitochondrial protein import and macromolecular assembly.	N.A.	N.A.
KRT1 414	3.2	Down	0.004	0.93	0.04	111	Keratin type-1, (cytoplasm or membrane bound)	Regulate activity of kinases via binding to integrin beta-1 and the receptor of activated protein kinase C (RACK1/GNB2L1).	Endothelial cell: complement activation, fibrinolysis, angiogenesis and response to oxidative stress.	(394)
212	3.5	Down	0.02	0.96	0.04	126				
ALB 341	8.8	Down	0.003	>0.99	0.02	85	Albumin (extracellular space)	Transporter; regulation of the colloidal osmotic pressure	Virology: down regulated in acute dengue virus infection.	(396)
PUUV infected versus Non-infected HUVEC and Lepto infected HUVEC										
PDIA3 441	2.2	UP	0.002	>0.99	0.2	807 (+118)	Protein disulfide isomerase family A. member 3, (cytoplasm)	Peptidase, Modulate folding of newly synthesized glycoproteins	Virology: MHC complex formation, dengue virus replication, $\alpha\text{V}\beta 3$ binding (Puumala virus receptor), host factor required for HIV infection. Endothelial cell: thrombus formation, Tissue factor activation.	(397-403)
NAPA 930	1.6	UP	0.001	0.99	0.1	47 (+39)	N-ethylmaleimide-sensitive factor attachment protein. Alpha (cytoplasm)	Transporter, Required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus	Virology: BK virus attachment, crucial in docking and fusion of vesicles to target membranes.	(404-406)
LAP3 447	3.0	UP	0.0001	>0.99	0.03	228	Leucine aminopeptidase 3 (cytoplasm)	Peptidase, Processing and regular turnover of intracellular proteins. Catalyzes the removal of unsubstituted N-terminal amino acids from various peptides	Virology: Elevated during herpes infection (CMV, EBV) and hepatitis C.	(407)
PRPF19 444	2.3	UP	0.0005	>0.99	0.07	149	Pre-mRNA processing factor 19 (nucleus)	Enzyme, Plays a role in DNA double-strand break	Endothelial cell: Increased expression leads to decreased apoptosis and increases cell survival.	(408)

								(DSB) repair, Structural component of the nuclear framework		
HNRNPK IFIT3 VIM NUCB1* 439	3.8	UP	0.0007	>0.99	0.08	101 86 83 68	Heterogeneous nuclear ribonucleoprotein K (Interferon-induced protein, Vimentin, Nucleobinin-1)	One of the major pre-mRNA-binding proteins	Virology: viral RNA splicing, pro-viral functions, interaction with human herpes 6, important in Enterovirus 71 virus replication, supports vesicular stomatitis virus replication.	(409-413)
PUUV infected versus Non infected HUVEC										
HSP90B1 108	1.8	UP	0.0017	>0.99	0.1	865 (+178)	Heat shock protein 90kDa beta member 1, (cytoplasm)	Molecular chaperone functions in processing & transport of secreted proteins	Virology: rotavirus replication, increased during vesicular stomatitis virus infection, hepatitis B virus replication biomarker. Endothelial cell: increased during apoptosis.	(414-418)
WARS 454	2.2	UP	0.0001	>0.99	0.03	102	Tryptophanyl-tRNA synthetase (cytoplasm)	Enzyme, eNOS activation. endothelial cell shear stress response. cytoskeletal reorganization	Virology: (Retro)virus expression, IFN response, increased during influenza (H3N2) infection.	(419-421)
SEPHS1 631	1.5	UP	0.003	0.97	0.2	78	Selenide. water dikinase 1	Enzyme, Synthesizes selenophosphate from selenide and ATP	N.A.	N.A.
HSPA5 252	3.0	Up	0.00018	>0.99	0.04	630	heat shock 70kDa protein 5 (cytoplasm)	Enzyme, assembly of multimeric protein complexes inside the endoplasmic reticulum. correct folding of proteins and degradation of misfolded protein	Virology: contributes to Ebola and Marburg virus infection <i>in vitro</i> , important for the release of dengue virus particles	(422;423)
ALDH1A1 425	1.5	Up	0.004	0.95	0.2	122 (+24)	Aldehyde dehydrogenase 1 family. member A1, (cytoplasm)	Enzyme, Binds free retinal and cellular retinol-binding protein-bound	Virology: increased during influenza (H3N2) virus infection. Endothelial cell: essential for retinoic acid production.	(421;424)
ALB 341	8.8	Down	0.01	0.9	0.2	85	Albumine (extra cellular space)	Transporter, regulation of the colloidal osmotic pressure	Virology: down regulated during acute dengue virus infection	(396)

Table 1. Identified proteins and the fold change of expression in the proteome of Puumala (PUUV), *Leptospira* and mock infected HUVEC with a subsequent review of the literature. Protein scores greater than 70 are significant ($p < 0.05$). In the 'score' column, score for individual peptides identified manually is added between brackets. * In spot 439 multiple proteins were identified and therefore it is not possible to conclude which protein is responsible for the increased abundance measured in the spot.

Functional pathway analysis of identified proteins

The IPA[®] analysis of the five proteins identified to be altered between infected and non-infected HUVEC lead to the identification of the following top-3, statistically significant, pathways in molecular and cellular functions: 1) Cellular assembly and organization, 2) cellular function and maintenance and 3) cell-to-cell signaling and interaction. Four of the five proteins are involved in the proliferation of cells ($p = 4.51e-03$; activation z score = -2) and in the migration of cells ($p = 1.3e-03$; activation z score = -0.6). Table 2 lists the top pathways identified after PUUV infection that are known pathways in diseases and disorders and the molecular and cellular functions involved.

Group	Molecules	P-value	Genes involved
<u>Diseases and disorders</u>			
<i>Hematological disease</i>	7	1.72E-06 – 4.26E-02	<i>Alb, HSPA5, PDIA3, ALDH1A1, HSPD1, KRT1, VIM</i>
<i>Immunological disease</i>	10	1.72E-06 – 2.53E-02	<i>ALB, HSPA5, KRT1, NAPA, PDIA3, VIM, ALDH1A1, LAP3, HSP90B1, HSPD1</i>
<i>Inflammatory disease</i>	10	1.72E-06 – 3.01E-02	<i>ALB, HSPA5, PDIA3, HSP90B1, HSPD1, LAP3, PDIA3, VIM, KRT1, ALDH1A1</i>
<i>Inflammatory response</i>	9	1.72E-06 – 4.52E-02	<i>ALB, HSPA5, PDIA3, HSP90B1, KRT1, NAPA, VIM, HSPD1, HSPB1</i>
<i>Respiratory disease</i>	7	1.72E-06 – 3.39E-02	<i>HSP90B1, HSPA5, ALB, PDIA3, HSPD1, PRPF19, VIM</i>
<u>Molecular and Cellular functions</u>			
<i>Protein synthesis</i>	10	6.98E-07 – 4.30 E-02	<i>HSP90B1, HSPA5, HSPB1, HSPD1, LAP3, PDIA3, WARS, KRT1, ALB, ALDH1A1</i>
<i>Cellular comprise</i>	5	1.71E-06 – 1.71E-02	<i>HSP90B1, HSPA5, HSPD1, VIM, ALB</i>
<i>Cellular function and maintenance</i>	8	1.71E-06 – 3.85E-02	<i>HSP90B1, HSPA5, VIM, HSPB1, NAPA, ALB, HSPD1, PDIA3</i>
<i>Cell death & survival</i>	10	4.00E-06 – 4.75E-02	<i>ALB, ALDH1A1, HSP90B1, HSPA5, HSPB1, HSPD1, NAPA, PDIA3, PRF19, VIM</i>
<i>Cell growth & proliferation</i>	11	1.40E-05 – 4.52E-02	<i>ALB, ALDH1A1, HSP90B1, HSPA5, HSPB1, LAP3, PDIA3, PRPF19, VIM, WARS</i>

Table 2. Functional groups of proteins with significant differences in abundance in HUVEC infected with PUUV compared to uninfected HUVEC. A total of 12 proteins (11 up-regulated; 1 down regulated) were mapped to functional groups using ingenuity IPA[®]. The functional groups with the highest significance, p-values and numbers of molecules of the groups of diseases and disorders and molecular and cellular functions are shown.

Discussion

Rodent-borne haemorrhagic fevers caused by either PUUV or leptospiral infection, occur with high incidence in humans across the world, causing significant morbidity and mortality. Both infections cause a very similar clinical picture in humans, often accompanied by renal failure and respiratory problems. A clear understanding of the pathogenic mechanisms of both PUUV infection and leptospirosis is lacking (62;210). Based on clinical observations and earlier *in vitro* work, it is likely that endothelial cell responses to infection with PUUV or *Leptospira* play a key role in the pathogenicity of both diseases. So far, few studies have examined host cell alterations following infection with either *Leptospira* or hantavirus (210;425). These studies mainly focused on microarray analysis of the transcriptome of infected cells. To date no studies analyzed the interplay between hantavirus and host cells using proteomic analysis, while for *Leptospira*, proteomic studies were performed mainly studying the pathogen, rather than the host, or serum from infected patients (426;427)

The aim of this study was to test the hypothesis that overlapping proteome changes in endothelial cells infected with a virulent low-passage isolate of a pathogenic hantavirus and a virulent human isolate of a pathogenic *Leptospira* occur. To this end we comparatively analyzed the proteomes of primary isolates of immune competent human endothelial cells infected with either PUUV or *Leptospira*.

We previously concluded that low passage PUUV isolates efficiently infected and replicated in the HUVEC primary cell culture (Goeijenbier *et al.*, manuscript under review). Immune peroxidase staining showed the presence of intracellular PUUV nucleoprotein with the amount of infected cells reaching 90% after 48 hours. Interestingly, leptospiral DNA, as quantified by qRT-PCR, increased in the cell lysate over time. Taken in mind that there was a clear intracellular staining positive for the presence of *Leptospira* this suggests the ability of the leptospires to move intracellular, something that has been suggested before, and possibly even replicate intracellularly. This observation could be interesting for future studies on potential survival mechanisms of the leptospires *in vivo*, but also aligns with our approach to compare the proteome of infected HUVEC after intracellular bacterial and viral infection.

All proteins with decreased abundance in PUUV infected HUVEC were also decreased in the proteome of cells infected with *Leptospira* (Figure 5B) which could suggest down regulation of proteins as a common pathway in the origin of endothelial dysfunction. Most of the five proteins identified by mass spectrometry, in the analysis between infected and non-infected HUVEC, are predicted to be cytoplasmic proteins, which aligns with the usage of intra cellular pathogens. For instance mass spectrometry identified the cytoplasmic protein vimentin as more than 5-fold down regulated protein after infection. Vimentin plays an important role in endothelial cell adhesion and sprouting. Regulation of this specific protein is driven by

posttranslational modifications such as phosphorylation and cleavage by intracellular proteases (394). Decreased functioning of vimentin leads to defects in (endothelial) cell attachment, migration, signaling and vascularization (394). Since it has been shown that the migratory function of endothelial cells decreases during hantavirus infection, the exact role of vimentin in this pathogenic process should be examined in greater detail, especially since the decrease of vimentin occurred in both infections of pathogens that cause endothelial cell dysfunction *in vivo*.

All proteins with decreased abundance in PUUV infected HUVEC were also decreased in cells infected with *Leptospira* (Figure 5B) which could mean that down regulation of these proteins is a common pathway in the origin of endothelial dysfunction.

Furthermore, our experimental design made it possible to use the bacterial infection as an extra control condition for PUUV infection and specifically study the host proteome changes in hantavirus infected endothelial cells; this is of great interest since hantaviruses are the only known viruses that specifically target endothelial cells (62). Some of the proteins with increased abundance after hantavirus infection, including “Protein disulfide isomerase family A member 3, Heterogeneous nuclear ribonucleoprotein K and Heat shock protein 90kDa beta member 1” are proven to be essential, or important, host factors for the replication of certain viruses as shown in Table 1. In spot 439 multiple proteins were identified and therefore it is not possible to conclude which of these proteins abundance was changed or if multiple proteins contributed to the increased fluorescence. A logical explanation would be the increase in Interferon-induced protein with tetratricopeptide repeats 3 (IFIT3) since HUVEC are immune competent cells and upon viral infection would respond by interferon production. However also heterogeneous nuclear ribonucleoprotein K (HnRNP k) has been associated with intracellular processes of several viruses. Dengue virus core proteins bind HnRNP k and thereby alters the function of the protein (413). Furthermore HnRNP k stimulates vesicular stomatitis virus replication, which, like PUUV is a negative stranded RNA virus, and seems also play a role in positive strand RNA virus replication; like hepatitis C and Enterovirus 71 (412;428;429). Interestingly HnRNP k interacts with vimentin and the intermediate filaments of vimentin are essential for nuclear HnRNP k expression and in this combination both proteins are essential for dengue virus replication and the release of virus particles (430). The decreased levels of vimentin during PUUV infection seem rather contradictory to this hypothesis. However, since an immune competent cell line was used, cell defense response upon PUUV infection could result in unexpected changes of the expression of these proteins (431;432). Based on our observations these proteins are excellent candidates for future studies on inhibition of PUUV replication involving siRNA or other knock out experiments exploring their potential role in hantavirus or more general negative stranded RNA virus replication. Another interesting observation that could be a lead for future studies is the more than 2-fold increase of protein disulfide isomerase (PDIA3). PDIA3 is important in thrombus formation and its capture in a

thrombus depends on beta3 integrins (400;401). Hantavirus *in vitro* studies showed a drastic up regulation of beta3 integrins, also known to be the receptor for hantavirus infection, during endothelial cell infection with different pathogenic hantaviruses (62;360;365). Furthermore PDIA3 seems to be a trigger for tissue factor dependent fibrin generation, a process which we recently showed to be heavily increased during HUVEC infection with PUUV (Goeijenbier et al. manuscript submitted) (402). No leptospiral or viral proteins were identified by mass spectrometry analysis. Of interest are three spots which were the most highly up regulated proteins in the PUUV infected endothelial cells (Table S1 spot 477, 486 and 746) that could not be identified but are highly likely to be of viral origin. Increasing the protein load from HUVEC infected cells with the respective pathogen on the gel for spot picking could overcome this shortcoming in the future.

In summary, we identified a number of proteins from primary cultured HUVEC with changed abundance during PUUV and *Leptospira* infection. Many of these proteins are important factors in cell maintenance and functioning while also at least five of them have been proven to play an essential role in the replication of specific viruses. Future studies should focus on knock down and elimination experiments to increase the understanding of the pathogenic mechanisms behind PUUV and leptospiral infection.

CHAPTER 3.3

Markers of endothelial cell activation and immune activation are increased in patients with severe leptospirosis and associated with disease severity

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Submitted

Abstract

Objectives: Previous studies concluded that haemorrhage is one of the most accurate prognostic factors of mortality in leptospirosis. Therefore, endothelial cell activation was investigated in relation to disease severity in severe leptospirosis. **Methods:** Prospective cohort study of severe leptospirosis patients. Plasma levels of sE-selectin and Von Willebrand factor (VWF) were determined. Consequently, an in vitro endothelial cell model was used to assess endothelial activation after exposure to virulent *Leptospira*. Finally, immune activation, as a potential contributing factor to endothelial cell activation, was determined by soluble IL2-receptor (sIL-2r) and soluble Fas-ligand (sFasL) levels.

Results: Plasma levels of sE-selectin and VWF strongly increased in patients compared to healthy controls. Furthermore, sE-selectin was significantly elevated (203 ng/ml vs. 157 ng/ml, $p < 0.05$) in survivors compared to non-survivors. Endothelial cells exposed to virulent *Leptospira* showed increased VWF expression. E-selectin and ICAM-1 expression did not change. Immunohistochemistry revealed the presence of intracellular *Leptospira* and qPCR suggested replication. In vivo analysis showed that increased levels of sFasL and sIL-2r were both strongly associated with mortality. Furthermore sIL-2r levels were increased in patients that developed bleeding and significantly correlated to duration of hospital stay.

Discussion: Markers of endothelial activation and immune activation were associated with disease severity in leptospirosis patients.

Introduction

Leptospirosis is an infectious disease of global importance (297). The disease is caused by spirochetes that are spread by the urine of infected animals. Mucous membranes, small cuts and abraded skin are the usual points of entry. Although the clinical course varies widely, leptospirosis can be fatal in up to 85% of all cases, especially in those that develop (pulmonary) haemorrhage (433;434). The more common mild form of leptospirosis is characterized by non-specific symptoms such as: acute fever, headache, chills, myalgia and conjunctival suffusion. In most severe forms, the clinical picture of leptospirosis may encompass jaundice, renal failure and haemorrhaging of skin, mucous membranes and/or lungs. Post mortem pathological findings confirm widespread haemorrhaging and endothelial cell dysfunction resulting in generalized oedema (297;435).

Clinical data from severe leptospirosis patients show significant activation of coagulation, which in many cases is followed by (often fatal) haemorrhage (381;382). Although the pathophysiology of the haemorrhagic diathesis in leptospirosis remains unclear, bleeding could very well be the result of endothelial cell dysfunction, since the endothelium is the key player in regulation of haemostasis (9). Under physiological conditions the vascular endothelium inhibits coagulation, prevents platelet aggregation and due to low levels of expressed adhesion molecules, it precludes adherence and migration of leukocytes. Injury or activation of endothelial cells in response to pathogens or to inflammatory cytokines can cause bleeding due to the loss of integrity of the blood vessel resulting in consumptive coagulopathy and/or vascular leakage (436). Thus far, *in vitro* work showed the ability of *Leptospira* to transmigrate through endothelial cell monolayers (210). Furthermore, an increase in adhesion molecules on the surface of human umbilical vein endothelial cells (HUVEC) was measured, when cells were incubated with *Escherichia coli* expressing leptospiral outer membrane proteins or with a recombinant leptospiral lipoprotein (383;384).

However, the exact role for endothelial cell activation, or source of damage, in the pathogenesis of leptospirosis remains unclear. In particular, it is not known whether haemorrhage is a direct function from exposure to the pathogen, or indirectly via host response factors (437). The aim of this work was to investigate endothelial cell activation in relation to bleeding in patients suffering from severe leptospirosis using a combined *in vivo*, *in vitro*, *in vivo* approach. To do so, we first determined the state of the endothelium in patients with severe leptospirosis, using soluble E-selectin (sE-selectin), a marker of activity expressed exclusively on the surface of endothelial cells (438) (438) and von Willebrand factor (VWF), also a sensitive marker of endothelial cell activation. We then evaluated the interaction between virulent *Leptospira* and endothelial cells *in vitro* using HUVEC. Combined, these results led us to hypothesise that T-cells play a more prominent role in the pathogenesis of severe leptospirosis. This hypothesis was tested in patients with severe leptospirosis by measuring the T-cell

activation marker soluble IL2-receptor (sIL2-r) and the immune mediated cell damage marker soluble Fas Ligand (sFas).

Materials and methods

Patients and controls

Consecutive patients with severe leptospirosis were included from February 2005 till September 2006 at the Dr. Kariadi hospital, Semarang, Indonesia. Severe leptospirosis was defined as hospitalized patients with a high clinical suspicion of leptospirosis, presenting with at least one of the following symptoms or signs: jaundice, renal failure, thrombocytopenia and/or bleeding and a positive LeptoTek Dri-Dot assay (Biomérieux), confirmed by microscopic agglutination test (MAT). We defined bleeding as the spontaneous occurrence of: petechiae, ecchymosis, epistaxis, gum bleeding, haematuria, melena, haematemesis and/or haemoptysis. After written informed consent was given, blood samples were taken on admission and during follow up at day 1, 2, 7 and 14. Citrated blood was centrifuged immediately and plasma aliquots were stored at -70°C until further analyses. As controls, 20 healthy Indonesian (Javanese) volunteers (no fever, no complaints at time of blood withdrawal) were tested. The medical ethics committee of the Dr. Kariadi hospital approved the study protocol.

Leptospirosis diagnosis

Clinical diagnosis of leptospirosis was confirmed by MAT and IgM enzyme linked immunosorbent assay (ELISA). For the MAT a panel of 31 serovars was used (28 pathogenic serovars and 3 non-pathogenic serovars). A titre of $\geq 1:320$ on a single sample, seroconversion or at least fourfold titre rise in paired samples or a titre $\geq 1:80$ in a single sample from early deceased patients, were considered to be positive.

Soluble E-Selectin and Von Willebrand Factor

Both endothelial markers, sE-selectin (R&D Systems, Minneapolis, MN, USA) and VWF (antibodies from Dako, Glostrup, Denmark) were determined by ELISA on citrated plasma samples.

Cells

Human umbilical vein endothelial cells (HUVEC) were harvested from fresh umbilical veins from healthy mother and baby. Isolated cells were de-identified and therefore could not be traced back to the original donor. Umbilical cords were stored in sterile 500 ml PBS + gentamycin (50 $\mu\text{g/ml}$) (Leo Pharmaceutical Products, Denmark). The veins were rinsed with PBS containing 50 U/ml heparin (Leo Pharmaceutical Products). Subsequently, cells were detached with 0.1%

collagenase solution (C6885, Sigma Aldrich, USA). Cell suspension was collected in a sterile 50 ml tube followed by centrifugation (2 x 5 minutes; 300 x g). The cell pellet was re-suspended in HUVEC medium (human endothelial-SFM medium, Invitrogen, USA) containing 10% human serum (Lonza, the Netherlands), 20% filtrated Fetal Bovine Serum (FBS); penicillin/streptomycin 100 U/ml, 20 ng/ml fibroblast growth factor (Peprotech, USA) and 10 ng/ml of endothelial cell growth factor (Peprotech). Cell suspensions were cultured in flasks pre-coated with 20 µg/ml of fibronectin (Roche, the Netherlands). Passage two cells from one specific donor were used for this study. The identity of the endothelial cells was confirmed by flow cytometry using *Ulex europaeus* lectin (EY laboratories, USA), anti-CD31 antibody (Sigma Aldrich, USA) and Von Willebrand Factor staining (Dako, the Netherlands).

Bacteria and infection

For the *in vitro* experiments the highly virulent, low passage isolate *L. interrogans* serovar Copenhageni (strain RJ16441) was used (henceforth denoted as: “virulent strain”). This strain was originally isolated from blood cultures of a patient with leptospirosis pulmonary haemorrhage syndrome as described in (387). The organism was maintained in Ellinghausen-McCollough-Johnson-Harris (EMJH) semisolid medium (EMJH medium containing 0.2% noble agar and 200 µg/ml 5-fluorouracil) or liquid culture medium (EMJH medium with 6% rabbit serum) at 30°C under aerobic conditions and regularly passaged through guinea pigs to preserve its virulence (for which ethical approval was given by the University College Dublin). *Leptospira* were harvested at the log phase of their growth ($1-2 \times 10^8$ /ml) and washed 2 times in RPMI medium before being re-suspended in HUVEC medium. For the infection 2.4×10^5 endothelial cells were incubated with a multiplicity of infection (MOI) of 10 either with the virulent pathogenic strain, or a multi-passaged non-virulent pathogenic, *Leptospira interrogans* serovar Copenhageni strain Wijnberg isolate (henceforth denoted as: “non-virulent strain”). The negative control consisting of bacteria free normal HUVEC medium (without antibiotics, with 20% FBS and no human plasma). After 24 and 48 hours of incubation, HUVEC were washed three times with PBS and formalin (no cell permeabilization) or methanol (with cell permeabilization) fixed.

Infection kinetics

For the evaluation of the infection kinetics of viable *Leptospira* in the endothelial cell model, we used an in house developed immune peroxidase staining procedure (*Goeijenbier et al. manuscript submitted*). Briefly, HUVEC were washed three times with PBS. Cells were fixed with absolute methanol (-20°C) for permeabilization at -20°C for 30 minutes or with 4% formalin for no permeabilization and incubated at room temperature for 30 minutes. After fixation, fixative was discarded and cells were incubated for 30 minutes at 37°C with 100 µl of 0.05% H₂O₂ in

PBS, to block endogenous peroxidases. Subsequently, cells were washed three times with PBS and incubated for 60 minutes with polyclonal rabbit anti-*Leptospira interrogans* serovar Copenhageni antibody (1:500, KIT Biomedical Research, the Netherlands). Cells were washed with PBS-0.05% Tween 20 followed by incubation with HRP-labelled goat anti-rabbit IgG conjugate (1:500; Dako, the Netherlands). Colour development was achieved by the addition of 3-amino-9-ethylcarbazole (AEC) substrate (AEC dissolved in dimethylformamide buffered with acetate buffer of pH 5). In addition, *Leptospira* were quantified using a real-time TaqMan® based quantitative PCR based on the *lipI32* gene for the quantitation of pathogenic *Leptospira* species. The DNA copy number per sample was calculated from a standard curve generated by a serial dilution of cultured *Leptospira* counted by dark-field microscopy with the use of a Helber counting chamber.

Von Willebrand factor, ICAM-1 and E-Selectin expression

HUVEC in 96 wells plates were fixed with 4% formalin and incubated with rabbit polyclonal anti-VWF antibody (1:500; Dako, Denmark), mouse monoclonal anti-ICAM-1 (1:500; Invitrogen, USA) or mouse monoclonal anti-E-selectin (1:500; Pierce antibodies, USA) followed by incubation with the respective conjugate (1:500; Dako). After washing TMB was added for substrate reaction and reaction was stopped after 10 minutes by addition of 0.5 M of sulphuric acid. Optical density at 450 nm value was read with Tecan ELISA reader.

Soluble Fas Ligand and soluble IL-2 receptor levels

T- cell activation marker sIL-2r and cell death marker soluble Fas ligand were measured in EDTA plasma samples using commercially available ELISA kits (Thermo Scientific, USA; Invitrogen, USA, respectively) according to the manufacturer's recommendations.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.01 for Windows. When comparing two groups we made use of a Student's t test or Mann Whitney U, depending on the distribution of the data. For the comparison between multiple groups, both for the *in vivo* and *in vitro* results, non-parametric Kruskal-Wallis test was used with Dunn's multiple comparison test or a one-way ANOVA with Tukey's multiple comparisons test, depending on the distribution of the data. P values ≤ 0.05 were considered significant.

Results

Endothelial cell activation in patients with severe leptospirosis

Patients

Fifty-two patients with severe leptospirosis were included in the study, of which 37 (71%) were male. The median age was 45 (IQR 33-55) years. Fourteen patients (27%) died during the course of infection. The median time from admission to death was 3 days. Bleeding was apparent in 31 (60%) patients; of these, 21 survived and 10 died (difference not significant). Signs of bleeding included: petechiae (n= 25), ecchymoses (n= 3), epistaxis (n= 2), melaena (n= 7), haematemesis (n= 2) and haematuria (n=1). On admission, 5 patients were stratified as non-septic, 28 as septic and 19 as severe septic. Statistically, bleeding was equally distributed among the non-septic, septic and severe septic patients. The median plasma creatinine and bilirubin levels were 5.4 mg/dl (IQR 3.1-7.6) and 8.3 mg/dl (IQR 3.4-20) respectively. Liver enzymes levels were only mildly elevated: ASAT 66 U/l, ALAT 53 U/l. Thrombocytopenia (platelets $\leq 100 \times 10^9/L$) was recognized in 32 (62%) subjects and was significantly associated with bleeding (OR 4.6, 95%CI: 1.3-16).

The endothelium is highly activated in patients with severe leptospirosis

Soluble E-selectin (mean: 169 ng/ml; SD 73) and VWF (mean: 500%; SD 182) levels in all 52 severe leptospirosis patients were strongly elevated on admission (Figure 1). The controls displayed significant lower mean values of sE-selectin (29 ng/ml; SD 9) and VWF (91%; SD 29) (Figure 1A & B). Both endothelial cell markers decreased gradually during follow up, but did not reach normal values by day 14 post admission. When comparing subgroups of patients with (n=31) and without (n=21) haemorrhagic manifestations on admission, no significant difference was observed in sE-selectin (175 ng/ml vs. 161 ng/ml; p = .50) or VWF (511% vs. 483%; p = .60) levels. However, subgroup analysis of patients that did not survive infection revealed a mean concentration of sE-selectin that was higher when compared to patients that survived (p < 0.05). Mean plasma levels of VWF were lower in the survivors (489%, SD 182) compared to non-survivors (530%,SD 186), but this difference did not reach statistical significance. In regard to disease severity, comparison of non-septic, septic and severe septic patients on admission and mean sE-selectin levels were: 136 ng/ml (SD 40), 186 ng/ml (SD 77) and 153 ng/ml (SD 70), respectively. Mean VWF levels were 432 % (SD 126), 538 % (SD 209) and 463 % (SD 141) respectively. These differences between non-septic, septic and severe septic patients were not significant.

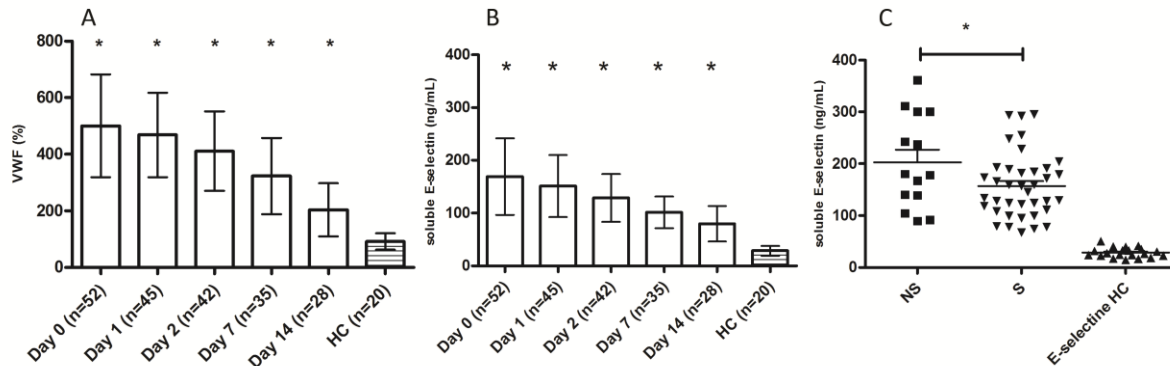


Figure 1. Plasma levels of soluble E-selectin (sE-Selectin) and von Willebrand factor (VWF) in patients with severe leptospirosis

VWF (A) and sE-selectin (B) plasma levels over time in patients suffering from severe leptospirosis (n = number of patients per timepoint). Results are presented as means with error bars indicating the standard error of the mean. The values denoted in the bars indicate the number of available samples. * p -value < 0.0001 (significant difference from controls). Soluble E-Selectin levels were increased in non-survivors at the day of hospital admission (day 0) (C; $p < 0.05$)

***In vitro* analysis of endothelial cell activation in leptospirosis**

Leptospira penetrate, survive and replicate within endothelial cells

While studying proteomic alterations of HUVEC infected with live pathogenic *Leptospira* (Goeijenbier et al. manuscript submitted), we noticed the ability of virulent *Leptospira* to persist within endothelial cells. To analyse the behaviour of *Leptospira* in the endothelial cell culture, using 10 *Leptospira* per endothelial cell, immune peroxidase staining was performed with and without cell permeabilization. Figure 2 shows positive staining of intra- and extra-cellular *Leptospira* in endothelial cells, 48 hours post infection. When cells were formalin fixed, only extracellular *Leptospira* were detected. Fixed cells with cell permeabilization (methanol fixed cells) revealed the presence of both intra- and extra-cellular *Leptospira*. Furthermore, quantitative real time PCR showed a statistically significant increase of total leptospiral DNA ($p < 0.05$). When the quantity of leptospiral DNA from the supernatant and cell lysate were added up, with the goal to quantify total leptospiral DNA, a significant increase was observed. In the first 48 hours after infection, leptospiral DNA increased from a mean of 6.3×10^4 (SD 1.2×10^4) copies to 1.85×10^5 (SD 2.6×10^4).

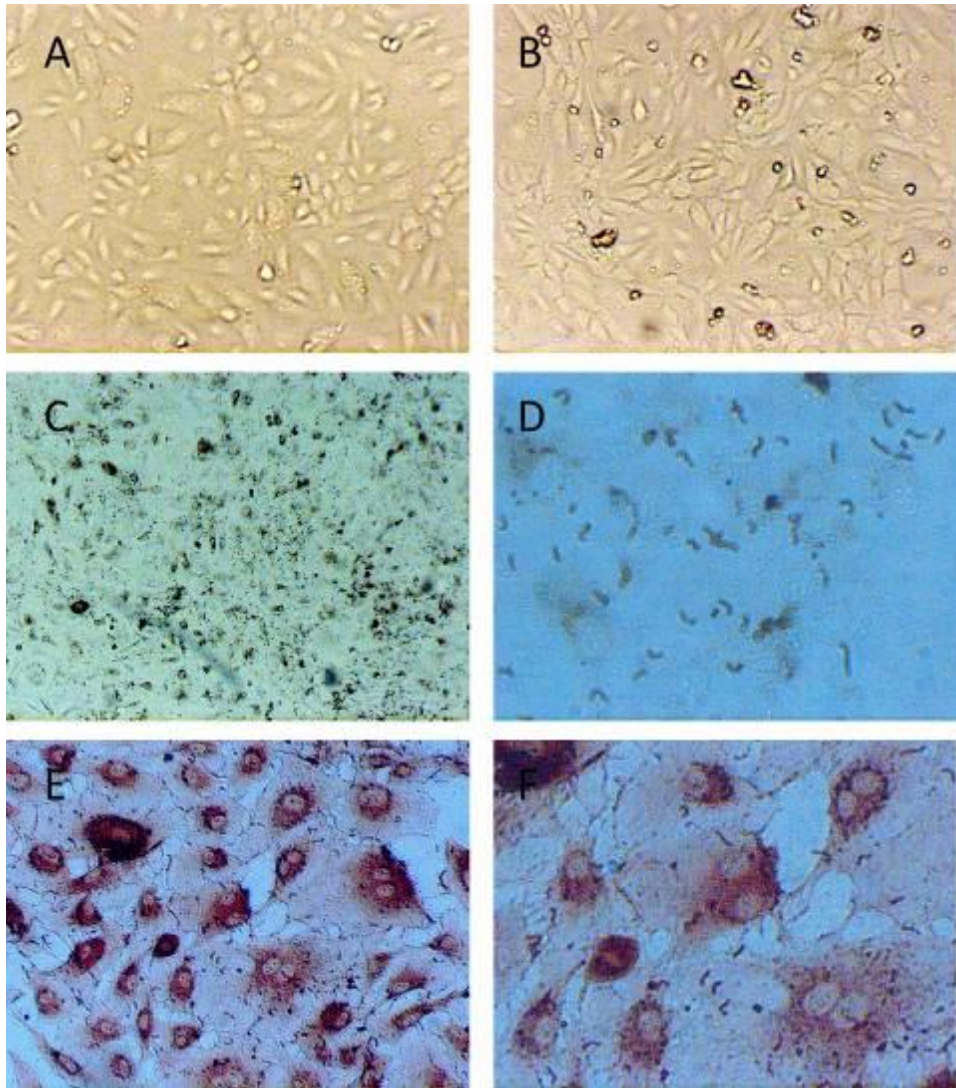


Figure 2. Immune peroxidase staining of HUVEC infected with *Leptospira*. The morphology of non-infected Human Umbilical Vein Endothelial cells (HUVEC) (2A) and HUVEC 48 hours incubated with a *Leptospira interrogans* multiplicity of infection of 10 (2B) is shown in the upper two panels and did not seem to differ between the two conditions. Panel C-F show the results of immunohistochemistry staining for the presence of extra cellular leptospira (C 10x & D 20x) and intra cellular *Leptospira* (E 10x & F 20x). After rigorous washing and cell permeabilization still clear intracellular staining is present in E and F suggesting the presence of at least *Leptospira* proteins inside the endothelial cells.

Virulent pathogenic Leptospira upregulate expression of VWF on the HUVEC surface of endothelial cells

To study if viable pathogenic *Leptospira* could directly increase markers of endothelial cell activation on the endothelial cell surface, we exposed HUVEC to virulent leptospires. To this end, 3×10^5 *Leptospira* (MOI 10) were incubated with endothelial cells. At 24 and 48 hours post infection, cells were formalin fixed and endothelial activation markers were quantified by incubation with a polyclonal antibody and HRP-labelled conjugate reaction. Both bacteria free medium and the non-virulent pathogenic *L. interrogans* serovar Copenhageni served as controls. Figure 3 shows the significant increase in VWF expression on HUVEC surface at 24 and 48 hours post infection with the virulent pathogenic *L. interrogans* serovar Copenhageni strain RJ16441 after the subtraction of blank optical density (OD) values and isotype control (rabbit polyclonal anti CD-3). The non-virulent pathogenic *L. interrogans* serovar Copenhageni strain Wijnberg was not able to induce significant VWF expression. Cell surface expression of E-selectin and ICAM-1 was not significantly changed at these time points when compared to the two control groups (data not shown).

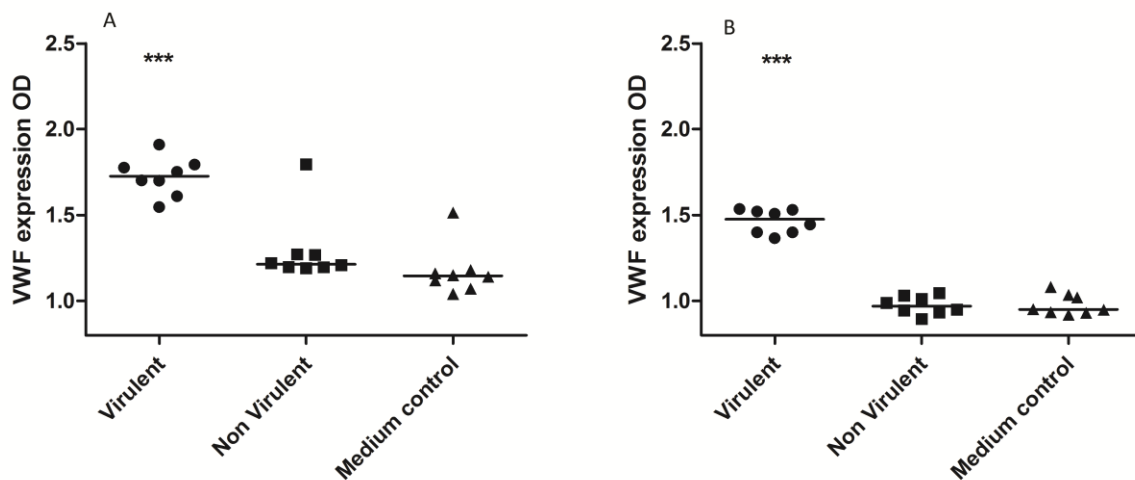


Figure 3. Cell surface quantification of von Willebrand factor (VWF) on infected and non-infected endothelial cells. Results of the cell surface quantification of VWF expressed by Optical Density (OD) after incubation with Von Willebrand antibody and matching conjugate after 24 (A) and 48 (B) hours of infection. Compared to both a non-virulent control of *Leptospira interrogans* and a bacteria free medium control the VWF OD expression is increased on HUVEC surface when incubated with live virulent *Leptospira interrogans* serovar Copenhageni ($p < 0.001$).

***In vivo* markers of immune activation and cell death in severe leptospirosis patients**

Soluble Interleukin 2 receptor levels are associated with survival and bleeding in severe leptospirosis patients

Since the *in vitro* experiments showed that virulent *Leptospira* were able to induce VWF expression on HUVEC, but not the endothelial cell activation markers E-selectin and ICAM-1, we aimed to study potential host factors in relation to endothelial cell dysfunction and consequently haemorrhage.

At first we tested plasma levels of the T-cell activation marker sIL-2r in the previous discussed cohort of severe leptospirosis patients. Admission sIL-2r levels were elevated with a mean value of 4700 U/mL (SD 3000 u/mL) and were significantly ($p < 0.001$) higher when compared to the healthy control group (Figure 4A), which showed a mean value of 600 U/mL (SD 200). Furthermore subgroup analysis showed that sIL-2r levels were significantly increased in patients that did not survive the infection ($p < 0.01$) (Figure 4B) and in those who developed bleeding during the course of infection ($p < 0.05$) (Figure 4C). The association between disease severity and sIL-2r levels was further confirmed by the statistically significant correlation between the number of days of hospital admission and sIL-2r levels at the day of admission (Spearman r 0.1-0.6; $p < 0.05$), when non-survivors were excluded from the analysis.

Plasma levels of sFAS Ligand are increased in severe leptospirosis patients that did not survive

To further assess the association between immune activation and disease severity in severe leptospirosis we tested the specific marker of immune mediated cell damage soluble Fas Ligand ligand. SFAS ligand was increased in non-survivors on the day of admission with a Log₁₀ mean value of 2.7 ng/mL (SD 0.4) compared to a Log₁₀ mean value of 1.9 ng/mL (SD 0.5) in survivors and 1.8 ng/mL (SD 0.3) in controls. On admission, the soluble Fas ligand (sFas ligand) plasma levels were significantly increased in non-survivors when compared to survivors ($p < 0.01$) and control patients ($p < 0.01$) (Figure 5). With a large variability in the data, no significant results were obtained when comparing bleeding to non-bleeding, or for correlations of markers of severe disease.

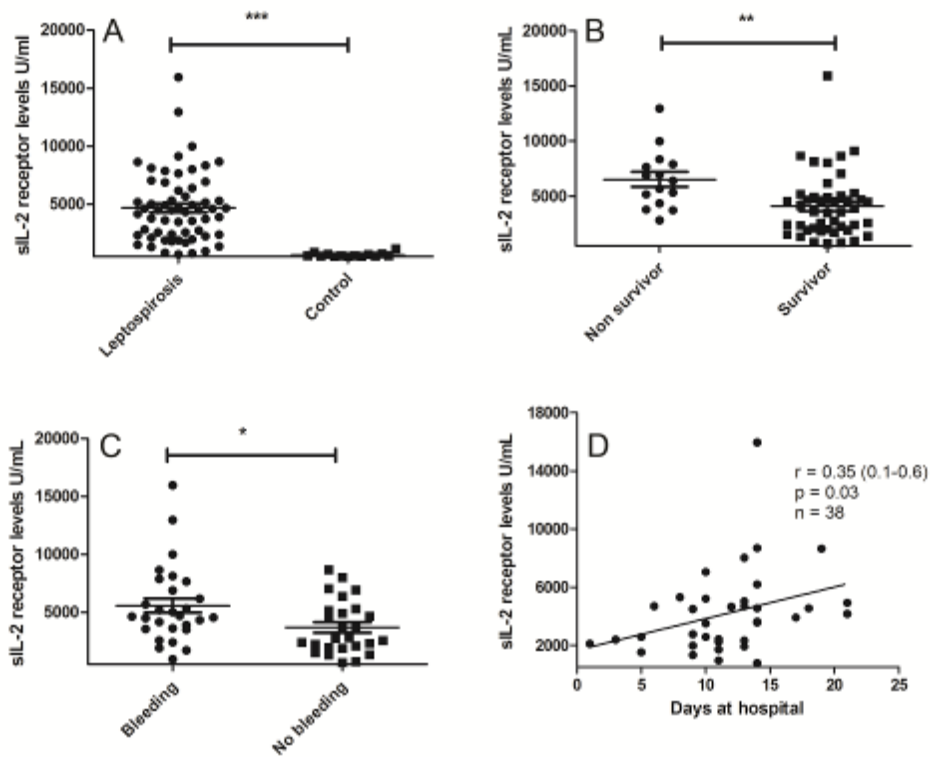


Figure 4. Soluble IL-2 receptor (sIL-2r) plasma levels in patients with severe leptospirosis. Levels of sIL-2r were significantly increased in severe leptospirosis patients at the day of hospital admission (4A; *** = $p < 0.001$). Furthermore, when performing subgroup analysis sIL2-r levels were higher in non-survivors compared to survivors (4B; ** = $p < 0.01$). The same holds true for patients that did develop bleeding and patients that did not (4C; * = $p < 0.05$). When correlating sIL2-r levels with days of hospital admission a significant correlation was seen with a Spearman r of 0.35 (4D; 0.1-0.6 95% CI $p = 0.03$)

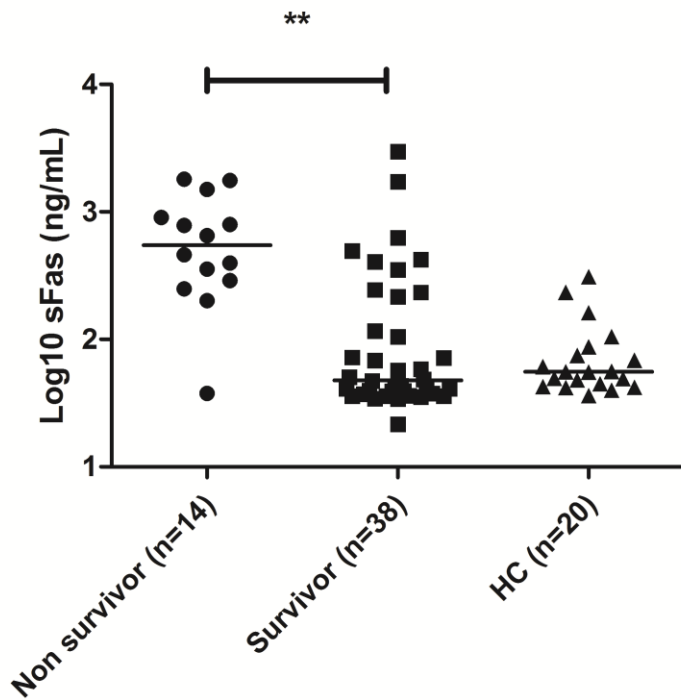


Figure 5. Soluble Fas ligand levels in severe leptospirosis patients.

*Patients that did not survive leptospirosis infection had higher sFAS ligand plasma levels at hospital admission than patients that survived or healthy controls (** $p < 0.01$). Between brackets are the number of included patients. HC stands for healthy controls. Bar represents the median.*

Discussion

This study aimed to investigate the role of endothelial cell activation and subsequent dysfunction in the pathophysiology of severe leptospirosis both *in vivo* and *in vitro*. At first, patterns of the endothelial cell activation markers sE-selectin and VWF were measured in patients with severe leptospirosis. Plasma levels of sE-selectin and VWF were highly increased in patients with severe leptospirosis ($p < 0.01$) reflecting considerable activation, or damage, of the endothelium. When comparing samples taken at the day of hospital admission, sE-selectin levels were significantly increased in patients that did not survive ($p < 0.05$). Although the association between mortality and the specific endothelial cell marker sE-selectin suggests a prominent role of the endothelium in the pathophysiology of severe leptospirosis, both VWF and sE-selectin levels were not significantly associated with bleeding in our cohort. These observations suggest that another factor also contributes to the bleeding diathesis in patients with severe leptospirosis. However, our assays were not able to distinguish between endothelial damage versus endothelial cell activation. Soluble E-selectin has its physiological activity on the endothelial cell surface, whereas VWF mediates adhesion of platelets to sites of vascular damage and is important for platelet aggregation by binding the GP IIb/IIIa platelet receptor. Both sE-selectin and VWF are released by endothelial cells when contacted with bacterial proteins, such as endotoxin(439;440). Intravenous administration of endotoxin into humans caused a dose dependent increase of sE-selectin, establishing its role as a quantitative marker of inflammation induced endothelial activation (439). The role of sE-selectin in the pathogenesis of leptospirosis could very well be comparable to the one that has been

extensively studied in bacterial sepsis where sE-selectin was proven to be an excellent marker for organ dysfunction (441). Activated endothelium can cause an imbalance in the ignition of the plasmatic coagulation pathways, which can lead to both thrombosis and bleeding. High levels of sE-selectin were observed in the present study, reflecting endothelial cell activation or damage. Furthermore, several studies have indicated that sE-selectin is elevated during septic conditions and that high levels are associated with poor clinical outcome (441) and therefore further analysis of this marker in leptospirosis is warranted. Elevated levels of VWF have been observed in several inflammatory disease states, including sepsis and septic shock. However data that correlate plasma levels of VWF with disease severity and patient outcome are inconsistent (441).

Secondly we addressed the capability of live, virulent *Leptospira* to activate primary culture endothelial cells *in vitro*, with the hypothesis that endothelial cell activation or damage could be a direct result of interactions of the pathogen with the host endothelium. Interestingly, infection with a MOI of 10 of the virulent *Leptospira* resulted in significant increased VWF cell surface expression when compared to cells incubated with bacteria free control medium or with a MOI 10 of culture attenuated non-virulent *Leptospira*. The specificity of this finding was supported by the fact that a non-virulent, multiple passaged control was used from the same serovar (Copenhageni). The activation markers E-selectin and ICAM-1 did not differ significantly after incubation with virulent *Leptospira* compared with the control groups, suggesting another mechanism than direct pathogen induced expression like we observed with VWF. However, our primary endothelial cell culture is limited to 48 hours of culture, while we must take note that leptospirosis patients have likely been exposed at least five days before showing symptoms, based on the mean incubation time of leptospirosis (179).

Thus far, data on the interaction between the endothelial cell and *Leptospira* are scarce. *Leptospira* were shown to induce endothelial cell adhesiveness for neutrophils in an experimental model, using HUVEC and sonicated *Leptospira* (442). Leptospiral peptidoglycan (which is not expressed on the surface of viable *Leptospira*) but not leptospiral LPS (which is surface exposed), actively induced the observed endothelial cell adhesiveness for neutrophils. Vieira et al. reported expression of E-selectin and ICAM-1 by HUVEC when directly stimulated with the leptospiral lipoprotein LIC10365 in a dose-dependent matter (384). Pathological studies suggest that the haemorrhagic phenomena in leptospirosis patients are due to capillary wall damage (435;443). One could speculate that *Leptospira* might be able to damage (endothelial) cell membranes directly or indirectly by production of toxins. It has been shown that pathogenic *Leptospira* contain different genes encoding for proteases and other products that can cause host cell membrane degradation like: sphingomyelinases and phospholipases (444). Of interest is the fact that VWF expression, but not E-selectin and ICAM-1, significantly increased on endothelial cells following incubation with live virulent *Leptospira*. Clearly endothelial cell responses to viable *Leptospira*, simulating a more physiological condition, differ

from endothelial cell responses to inactivated *Leptospira* or leptospiral proteins as was shown in previous studies. Furthermore, future research should include different strains of *Leptospira*, since *Leptospira* strains clearly differ in phenotype and even associated with a different clinical presentation when they infect human. Indeed, analysis of the infection kinetics and the viability of the virulent *Leptospira* in culture revealed intracellular *Leptospira* and increased leptospiral DNA over the time of infection underlining the importance of the interaction between viable bacteria and endothelial cells. Moreover results suggest alternative, indirect pathogenic mechanisms that lead to VWF expression, and other pathways that may result in endothelial dysfunction. Hence it was hypothesised that the presence of intra endothelial *Leptospira*, or *Leptospira* stuck to the cell surface of endothelial cells, may lead to endothelial cell activation or even damage as a bystander effect from a targeted immune response.

In the third part of the study, plasma levels of the B, but especially T cell activation marker sIL2-receptor and the immune mediated cell death marker sFas ligand were measured. Both markers showed significantly increased levels in those patients that did not survive infection. Furthermore sIL2-receptor levels were increased in patients that developed haemorrhage and positively correlated to the duration of hospital admission. Petro et al. demonstrated highly increased sIL-2r levels in a patient with severe leptospirosis admitted to the hospital in Germany and a possible correlation towards disease severity (445). This study confirms the relation between sIL-2r levels and disease severity in leptospirosis in a cohort. Furthermore, corticosteroid treatment, and subsequent T-cell suppression, might increase survival chances in severe pulmonary leptospirosis (446); future studies focussing on the role of T-cells in severe leptospirosis should be executed. The increased levels of sFas ligand in non-survivors in our cohort is of great interest for further discussion. First of all since sFas ligand seems to play an important role in the induction of acute lung injury and induces apoptosis in endothelial cells (447;448). Furthermore, increased levels of sFas ligand are associated with higher Sequential Organ Failure Assessment scores suggesting a potential role for this protein in bacterial sepsis (449). In our cohort sFas ligand plasma levels at the day of hospital admission seems an excellent prognostic factor for non-survival. However, the large variability in sFas ligand levels in our cohort, the relative small numbers of patients included and the absence of kinetic analysis of sFas ligand levels in all patients warrants larger (prospective) studies. Furthermore, one should take in mind that only patients with severe leptospirosis were enrolled in our study. In a previous study, sE-selectin was shown to be elevated in 20 Thai patients with mild leptospirosis (450). These levels were higher (albeit not significantly) compared to patients presenting with murine typhus, scrub typhus, typhoid, dengue fever or uncomplicated malaria but lower compared to our results. Of future interest would be the differentiation between endothelial cell activation and endothelial cell damage in (severe) leptospirosis. A better understanding of endothelial cell damage rather than activation would be of great interest, for instance by measuring circulating endothelial cells which have been shown to correlate to

disease severity in Haemorrhagic Fever with Renal syndrome, a disease with many clinical similarities with leptospirosis (358).

In conclusion, results demonstrate that markers of endothelial cell activation are strongly elevated in patients with severe leptospirosis. Furthermore, *in vitro* viable virulent *Leptospira* moved intracellular where they caused an increase in VWF expression on the cell surface of the endothelial cells. Furthermore, the T-cell activation marker sIL2-r and immune related cell damage marker sFas ligand were statistically significantly increased in severe leptospirosis patients and associated with mortality. Future studies are needed to establish the precise role of the endothelial cell in the pathophysiology of leptospirosis while prognostic clinical cohort studies should include whether the use of sIL-2r and sFas ligand as prognostic biomarkers in severe leptospirosis is of use.

CHAPTER 3.4

Activation of coagulation and tissue fibrin deposition in experimental influenza in ferrets

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Abstract

“Epidemiological studies relate influenza infection with vascular diseases including myocardial infarction. In several animal model studies the procoagulant effects of influenza virus infection have been investigated. Since these studies made use of animals only susceptible to laboratory animal adapted influenza viruses, their results are hard to translate to human influenza. Therefore we decided to study the influence of different human influenza virus infections on coagulation in the ferret influenza model. Ferrets were infected with either a seasonal-, pandemic- or highly pathogenic avian influenza (HPAI-H5N1) virus strains, or mock,infected. In a 14 day interval (only 4 days for HPAI-H5N1) with in total 7 time points, 4 animals were euthanized per timepoint and citrated plasma was tested for prothrombin time (PT), activated partial thromboplastin time (aPTT), Von Willebrand factor activity (VWF), thrombin-antithrombin complex levels and D-dimer. Lung tissue was used for fibrin staining. All influenza virus infected animals showed alterations in haemostasis. Specifically on day 4 post infection, a four second rise in both PT and aPTT was observed. D-dimer concentrations increased in all 3 influenza groups with the highest concentrations in the pandemic influenza group. Von Willebrand factor activity levels increased early in infection suggesting endothelial cell activation. Mean thrombin-antithrombin complex levels increased in both pandemic and HPAI-H5N1 virus infected ferrets. At tissue level, fibrin staining showed intracapillary fibrin deposition especially in HPAI-H5N1 virus infected ferrets. This study showed hemostatic alterations both at the circulatory and at the tissue level upon infection with different influenza viruses in an animal model closely mimicking human influenza virus infection. Alterations largely correlated with the severity of the respective influenza virus infections.”

Background

There are three manifestations of influenza in humans: seasonal, avian and pandemic influenza. Seasonal influenza is caused by influenza A or B viruses which infect 5-15% of the human population every year (66). Symptoms vary from mild respiratory complaints to fatal respiratory distress due to multiple organ failure. Symptoms depend largely, however, on the health and immune status of the infected individual and the pathogenicity of the specific virus involved. While avian influenza A viruses cause sporadic zoonotic infections in humans, that do not spread efficiently among humans (451), these infections may result in respiratory disease manifestations that range from mild to fatal, which among other variables largely depends on the virulence of the virus involved. Although most seasonal influenza virus infections are self-limiting, they do cause a considerable burden of disease that may be aggravated by complications of the infection (452). Patients with chronic illness are particularly at risk of developing these complications when suffering from (seasonal) influenza, like the observed increased risk for developing cardiovascular disease during or shortly after influenza virus infection (453). This observation is supported by the results of two intervention studies which showed a risk reduction of myocardial infarction after influenza vaccination, which later was confirmed by a meta-analysis carried out among 292,383 patients. This analysis showed significant reductions in myocardial infarction, all-cause mortality, and major adverse cardiac events in the influenza vaccinated groups (454-456). However, the etiological pathway and the frequency by which influenza predisposes for clinically relevant thrombotic disease has yet to be determined. Current data suggest that influenza virus infection causes an unbalanced coagulation manifested by a procoagulant state (for review see (20;80-82)). Indications for this increased clotting tendency have come from clinical, experimental mouse and *in vitro* data. Clinical reports range from mild increased coagulation and fibrinolysis markers such as von Willebrand factor (VWF) and D-dimer levels, to disseminated intravascular coagulation observed in severe avian influenza (83-85). Experimental mouse data indicate a procoagulant state characterized by increased thrombin generation, fibrin deposition, and an impaired fibrinolysis (86;87). However, as the mouse is not a natural host to influenza virus, mouse influenza models use mouse-adapted influenza viruses which cause a disease quite different from that of human influenza (457). Collectively experimental animal studies and epidemiological data have largely remained inconclusive and cannot readily be translated into clinically relevant conclusions.

The laboratory ferret (*Mustela putorius furo*) is not only susceptible to human isolates of seasonal, avian and pandemic influenza viruses, but pathogenesis and severity of the respective clinical manifestations of these infections are to a large extent similar to those found in humans (458;459). Therefore, to address the hypothesis that humans at risk for vascular disease may develop clinically overt vascular thrombosis during or shortly after influenza virus infection (460), we collected plasma samples during a time course pathogenesis experiment in which

ferrets were infected with seasonal-, avian- or pandemic influenza viruses (461). Even though ferrets are not generally considered to represent the high risk patients for vascular thrombotic disease, they do offer a biologically variable and reliable animal model to address the activation of coagulation during influenza virus infection. Prothrombin time, activated partial thromboplastin time, von Willebrand factor (VWF) activity, D-dimer levels, and thrombin-antithrombin complexes were measured in sequentially collected plasma samples. In addition fibrin staining was carried out on the lungs of infected animals upon euthanasia to address the coagulation status at the tissue level. All these parameters were evaluated in relation to virological parameters and data on disease severity.

Material & Methods

Experimental design

Samples from 104, 11-month old, male, outbred ferrets (*Mustela putorius furo*) were used for this experiment as described previously (461). Animals were inoculated both intratracheally and intranasally with one of three influenza viruses, or with control material (mock). All three influenza virus strains had been directly derived from patient isolates. For seasonal influenza, H3N2 virus (A/Netherlands/177/2008) (458), for pandemic influenza, pH1N1 influenza virus (A/Netherlands/602/2009) (462) and for highly pathogenic avian influenza virus (HPAI) the H5N1 strain (A/Indonesia/5/2005) were used (463). Virus stocks were passaged three times in Madin-Darby Canine Kidney (MDCK) cells and titrated according to standard methods. The viruses were clarified and reached an infectious virus titre of $10^{7.4}$ median tissue culture infectious dose (TCID₅₀) per ml for H3N2 virus, and $10^{7.8}$ TCID₅₀ for both pH1N1 and HPAI-H5N1 virus (464). The inoculum of the control group consisted of MDCK culture derived material which had been subjected to the same procedure to control for respiratory tract damage not related to replicating virus (461). Inocula consisted of 3 mL volumes of virus preparations with 10^6 TCID₅₀ given per animal partly intratracheally and partly intranasally. Ferrets were randomly selected for any of the predefined time points before the start of the experiment. Four ferrets were euthanized per time point. Each ferret was sampled twice: before inoculation and when sacrificed. This resulted in 104 samples analyzed before inoculation (28 mock, 28 H3N2, 28 pH1N1 and 20 H5N1) and 4 samples per virus per time point (Table 1). During euthanasia, citrated blood was drawn by cardiac puncture in 3mL citrate tubes and plasma was prepared for testing in coagulation assays.

Ethics statement

To reduce the numbers of experimental animals used, we combined the earlier published influenza pathogenesis study (461) with the current study addressing questions related to activation of coagulation and tissue fibrin deposition during influenza virus infection. Animal housing and experiments were all in compliance with European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997) as documented previously (461). The study protocol was approved by the independent animal experimentation ethical review committee of the Netherlands Vaccine Institute (permit number 200900201). Animal welfare was observed on a daily basis, and animal handling was performed under light anesthesia using a mixture of ketamine and medetomidine. After handling, atipamezole was administered to antagonize the effect of medetomidine.

Coagulation assays

Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using a BCS-XP coagulation analyzer (Siemens Healthcare Diagnostics) according to the instructions of the manufacturer. Clotting was initiated with Thromborel S (PT) and Pathrombin SL (APTT). VWF ristocetin cofactor activity was also determined on the BCS-XP with reagents of the manufacturer, and was expressed as percentage of normal pooled human plasma. Thrombin-antithrombin complexes (TAT, Siemens Healthcare Diagnostics) and D-dimer levels (Asserachrom, Roche, The Netherlands) were measured using enzyme-linked immunosorbent assay. All these assays were carried out within the BSL-3 setting after careful calibration and validation.

Pathology and fibrin staining

Gross pathology and histopathology were evaluated as previously described (461). Relative lung weight was used as a validated measure of gross pathology and lung inflammation (465). For detection of fibrin, tissues were stained with the Lendrum staining according manufacturers' protocol (MSB RRSK2-100 stain kit, Atom scientific). On each slide a small piece of human placenta was added as a positive control. Semi-quantitative assessment of fibrin expression in the lungs was performed as follows: for the alveoli, 25 arbitrarily chosen, 20x objective, fields of lung parenchyma of one lung section were examined by light microscopy for the presence of fibrin, without the knowledge of the identity of the animals. The scores (+ or -) were multiplied by 4 and presented as percentage.

Virology

The presence of virus and virus replication in the respiratory tract were measured by determining infectious virus titres at different sites of the upper respiratory tract (URT) and lower respiratory tract (LRT). These results were combined with data retrieved by measuring viral antigen expression using standardized semi-quantitative immunohistochemistry carried out at different sites of the LRT as described previously (461).

Statistics

All statistical analyses were performed using the software SPSS PASW statistics 17.0 and GraphPad Prism 4.01 for Windows. The data were expressed as mean or median with or without standard deviation or 95% confidence interval as described in figure and table legends. The compared groups are summarized in Table 1. The means per time point between the influenza virus infected groups and the mock control infected group were analyzed using the Mann-Whitney U test. Furthermore, values at the predefined time point of euthanasia were compared with pre-inoculation samples using paired t-testing. Differences with $p \leq 0.05$ were considered statistically significant. For comparison of individual association between virological parameters and coagulation markers we used Pearson correlation coefficient, and transformed to match a normal distribution if needed. For correlation analysis we used Bonferroni correction for multivariable comparison setting p-value threshold to $p \leq 0.01$.

RESULTS

Clinical signs, pathology and virology of ferrets after infection with H3N2-, pH1N1- or highly pathogenic H5N1 avian – influenza viruses

Clinical signs, pathological changes and virological parameters of this time course experiment in ferrets have been reported previously (461). Data important for this study are summarized in Table 2. In short, clinical signs varied greatly between the three influenza virus and mock infected groups. All animals infected with H3N2, pH1N1, or mock infection, survived the infections. H3N2 virus infected ferrets showed mild clinical signs; nasal discharge, sneezing, decreased tendency to eat, and bodyweight decrease by 11% (SD 8.5-13%) at 7 dpi. Detection of infectious virus was restricted to the nose and peaked at 1 dpi. Upon necropsy the lungs of the H3N2 infected ferrets showed up to 10% consolidation by gross pathology while the relative lung weights did not differ from the controls.

Ferrets infected with the pH1N1 virus showed more severe clinical signs compared to the seasonal H3N2 virus infected ferrets, with a body weight decrease around 15% (SD 11.4-18.6%). Viral titres during pH1N1 virus infection also peaked at 1 dpi, but occurred at similar levels throughout the whole respiratory tract. One ferret in the pH1N1 group developed severe

dyspnea. Relative lung weights increased compared to those of the mock infected animals starting from day 1. Their relative lung weights (weight of lung divided by bodyweight multiplied by 100) had increased from 0.6 % (SD 0.57-0.65) to 1.3 % (SD 1.0-1.6). The lungs of the pH1N1 virus infected ferrets showed up to 70% consolidation by gross pathology.

The HPAI-H5N1 virus infected ferrets showed more severe clinical signs with dyspnea leading to hypoxia. On 2.5 dpi, one animal died and one animal was euthanized for ethical reasons. On 3 dpi, another animal died before it could be euthanized. H5N1 virus was predominantly found in the alveoli and viral titres peaked for a longer period, from 1 to 3 dpi. Upon necropsy the lungs of the H5N1 infected ferrets showed up to 100% consolidation by gross pathology and relative lung weight was increased up to 2.78% of total body weight, while pre-inoculation samples had a mean relative lung weight of 0.66%. Mock infected ferrets showed no significant clinical signs or weight loss. Only minor consolidations in about 10% of the lung tissue were found upon necropsy.

To assess a potential link between hemostatic alterations with total virus titres we generated the areas under the curve (AUC) from the virus titre as shown in Table 3.

<i>Group</i>	<i>P.I.</i>	<i>½ dpi</i>	<i>1 dpi</i>	<i>2 dpi</i>	<i>3 dpi</i>	<i>4 dpi</i>	<i>7 dpi</i>	<i>14 dpi</i>	X
Mock	28	4	4	4	4	4	4	4	↓
H3N2	28	4	4	4	4	4	4	4	
pH1N1	28	4	4	4	4	4	4	4	
H5N1	20	4	4	4	4	4	0	0	
total	104	16	16	16	16	16	12	12	
Z									Y

Table 1. Distribution of the ferrets used in this study. Ferrets were sampled before inoculation with a mock control suspension, H3N2-, pH1N1- or H5N1 influenza virus. Coagulation analysis values from the different groups were compared using Mann-Whitney U test (X to Y). Subsequently values from the predefined timepoints were analyzed with the pre inoculation (P.I.) values using paired t-test (Y to Z).

Day		1	2	3	4	7	14
Bodyweight	H3N2	-51	-100	-69	-124	-186	-205
		(16-86)	(9-190)	(33-104)	(117-130)	(141-231)	(101-309)
	pH1N1	-68	-169	-142	-250	-251	-193
		(22-114)	(161-176)	(74-210)	(185-315)	(190-312)	(19-368)
	H5N1	-70	-131	-170	-190	†	†
		(35-105)	(112-149)	(142-198)	(135-246)		
Control	-44	-20	+7	-34	-62	-46	
	(31-57)	(+30 - -69)	(+40- -25)	(+19 - -88)	(+10 - -134)	(+30 - -123)	
Relative lung weight 10 ⁻² gram	H3N2	0.6	0.6	0.6	0.6	0.6	0.6
		(0.5-0.7)	(0.6-0.7)	(0.5-0.6)	(0.5-0.7)	(0.6-0.7)	(0.5-0.6)
	pH1N1	0.8	1.0	1.1	1.2	1.3	0.7
		(0.7-0.8)	(0.9-1.2)	(0.9-1.2)	(1.1-1.3)	(1.0-1.6)	(0.6-0.8)
	H5N1	0.9	1.4	1.7	2.4	†	†
		(0.6-1.2)	(1.1-1.7)	(1.2-2.2)	(2.0-2.7)		
Control	0.7	0.7	0.6	0.6	0.6	0.6	
	(0.7-0.8)	(0.6-0.8)	(0.6-0.6)	(0.6-0.7)	(0.6-0.7)	(0.5-0.7)	
Lung damage %	H3N2	3.8	2.5	0	0	0	1.3
		(0-8.5)	(0-5.4)				(0-3.8)
	pH1N1	22.5	25.0	40.0	45.0	47.5	25.0
		(17.5-27.5)	(19.2-30.8)	(31.8-48.1)	(35.0-55.0)	(30.4-64.6)	(19.2-30.8)
	H5N1	25.0	55.0	62.5	77.5	†	†
		(12.1-37.9)	(35.9-74.2)	(40.3-84.7)	(55.3-99.7)		
Control	3.8	6.3	6.3	1.3	5.0	3.8	
	(1.3-6.3)	(1.5-11)	(1.5-11)	(0-3.8)	(5-5)	(1.3-6.3)	
Turbinates/ nasal concha logTCID50	H3N2	7.0	6.3	5.1	4.8	neg	neg
		(5.5-8.5)	(5.4-7.3)	(3.9-6.2)	(3.4-6.1)		
	pH1N1	8.2	8.0	7.6	7.0	neg	neg
		(8.0-8.5)	(7.7-8.3)	(7.0-8.2)	(6.2-7.9)		
	H5N1	4.8	5.0	5.6	4.9	†	†
		(3.5-6.1)	(4.4-5.6)	(4.1-7.0)	(3.4-6.4)		
Trachea logTCID50	H3N2	2.4	neg	neg	neg	neg	neg
		(<1.7-3.1)					
	pH1N1	5.5	5.4	5.9	5.5	neg	neg
		(5.0-6.0)	(5.0-5.9)	(5.6-6.3)	(4.3-6.9)		
	H5N1	5.5	4.7	5.1	4.7	†	†
		(4.7-6.3)	(4.2-5.1)	(4.1-6.2)	(3.4-6.0)		
Lung logTCID50	H3N2	neg	Neg	Neg	neg	neg	neg
		pH1N1	7.5	5.2	5.5	5.6	neg
		(7.2-7.8)	(4.7-5.8)	(5.1-6.0)	(5.1-6.2)		
	H5N1	6.6 (6.0-7.2)	5.2 (4.7-5.6)	5.8 (5.5-6.1)	5.2 (4.7-5.6)	†	†

Table 2. Overview of the clinical data (bodyweight decrease, relative lung weight, lung damage) and Virological parameters.

<i>Virus</i>	<i>Day</i>	<i>Virus titre*</i>	<i>Lung virus AUC#</i>	<i>Respiratory tract AUC#</i>
H3N2	0.5	3.5 (2.9-4.2)	neg	0
	1	7.0 (5.5-8.5)	neg	2.6
	2	6.3 (5.4-7.3)	neg	9.3
	3	5.1 (3.9-6.2)	neg	15
	4	4.8 (3.4-6.1)	neg	19.9
pH1N1	0.5	26.0 (24.3-27.7)	0	0
	1	31.7 (31.1-32.3)	3.6	14.4
	2	27.0 (26.4-27.6)	10.0	43.8
	3	27.0 (25.7-28.4)	15.4	70.8
	4	25.7 (23.4-28.0)	20.1	97.1
H5N1	0.5	22.3 (19.5-25.2)	0	0
	1	27.61 (24.4-30.8)	3.1	12.5
	2	24.8 (22.3-27.3)	9.0	38.7
	3	26.1 (22.0-30.8)	14.5	64.3
	4	26.0 (23.9-28.0)	19.9	90.5

Table 3. Viral parameters for correlation tests with coagulation results from 0.5-4 dpi.

* Total virus titre in logTCID 50 (cumulative titres of all organs with significant virus titres: “lung, nasal concha, trachea, bronchus and bronchial lymph nodes”) (+/- SD) # AUC was calculated from virus titres curves. 7 dpi and 14 dpi were excluded from the analysis because we data points from 5 & 6 dpi are not available potentially resulting in over or underestimation of the true AUC

Both prothrombin time and activated partial thromboplastin time show transient prolongations during influenza virus infection in ferrets

To evaluate tissue factor pathway activation of the coagulation cascade we tested the prothrombin time (PT) for all samples. Before inoculation all ferrets had PTs within normal range.

Figure 1 (row A) summarizes the PT results over time for all four groups. For both the H3N2 virus and pH1N1 virus groups, PT values increased with approximately 4 seconds at 4 dpi compared to pre-inoculation samples (H3N2 $p = 0.001$, pH1N1 $p = 0.02$) and the mock infected animals at the same day (H3N2 $p = 0.03$, pH1N1 $p = 0.03$). In the H5N1 infected ferrets, PT prolongation started at 2 dpi with a prolongation up to 16 seconds in individual animals. A clear trend is seen with PT increasing up to 30 seconds at 3 dpi. On multiple occasions ferrets died before samples could be drawn, consequently the data depend on a small number of observations with a potentially strong survival bias. On 4 dpi only one sample met the quality criteria for PT testing in the H5N1 group with a PT of 13.4 seconds, a 1.4 second increase compared to mean + SD from day 0 and mock samples (+/- SD). No significant changes in PT were observed over time in the mock infected group. Row B in Figure 1 shows the Activated partial thromboplastin time (APTT) a measurement of the intrinsic pathway of coagulation. APTT's showed similar trends as PT's. At 4 dpi, APTT's were prolonged in all the three infected groups (Figure 1). H3N2 virus and pH1N1 virus infected ferrets showed mean APTT's of 27.8 (26.1-29.6) and 24 (19.6-28.4) seconds respectively while for the mock group this was 19.7 (18.5-20.9) seconds. Paired testing showed that the pH1N1 virus infected ferrets had significantly prolonged APTT's than the samples from pre inoculation ($p = 0.02$). No significant difference was seen compared to the mock infected group, potentially due to lack of power. Comparing 4 dpi samples with all pre-inoculation samples results in significant differences for both H3N2 and pH1N1 (H3N2 $p = 0.001$ pH1N1 = 0.02). Three out of four ferrets inoculated with H3N2 and sacrificed at 4 dpi already showed APTT prolongation before inoculation. This was not observed in any of the other pre-inoculation samples, but hampers the interpretation of the significant lengthening on 4 dpi compared to the mock infected group ($p = 0.03$) resulting in a non-significant result in paired sample testing. HPAI-H5N1 virus infected ferrets showed a trend toward prolonged APTT on 3 dpi with a mean of 28 (17.1-38.9) seconds and on 4 dpi 26.3 (17.3-25.3) seconds, which was statistically significant when compared to all APTT results in pre inoculation samples (3 dpi $p = 0.02$,4 dpi $p = 0.02$).

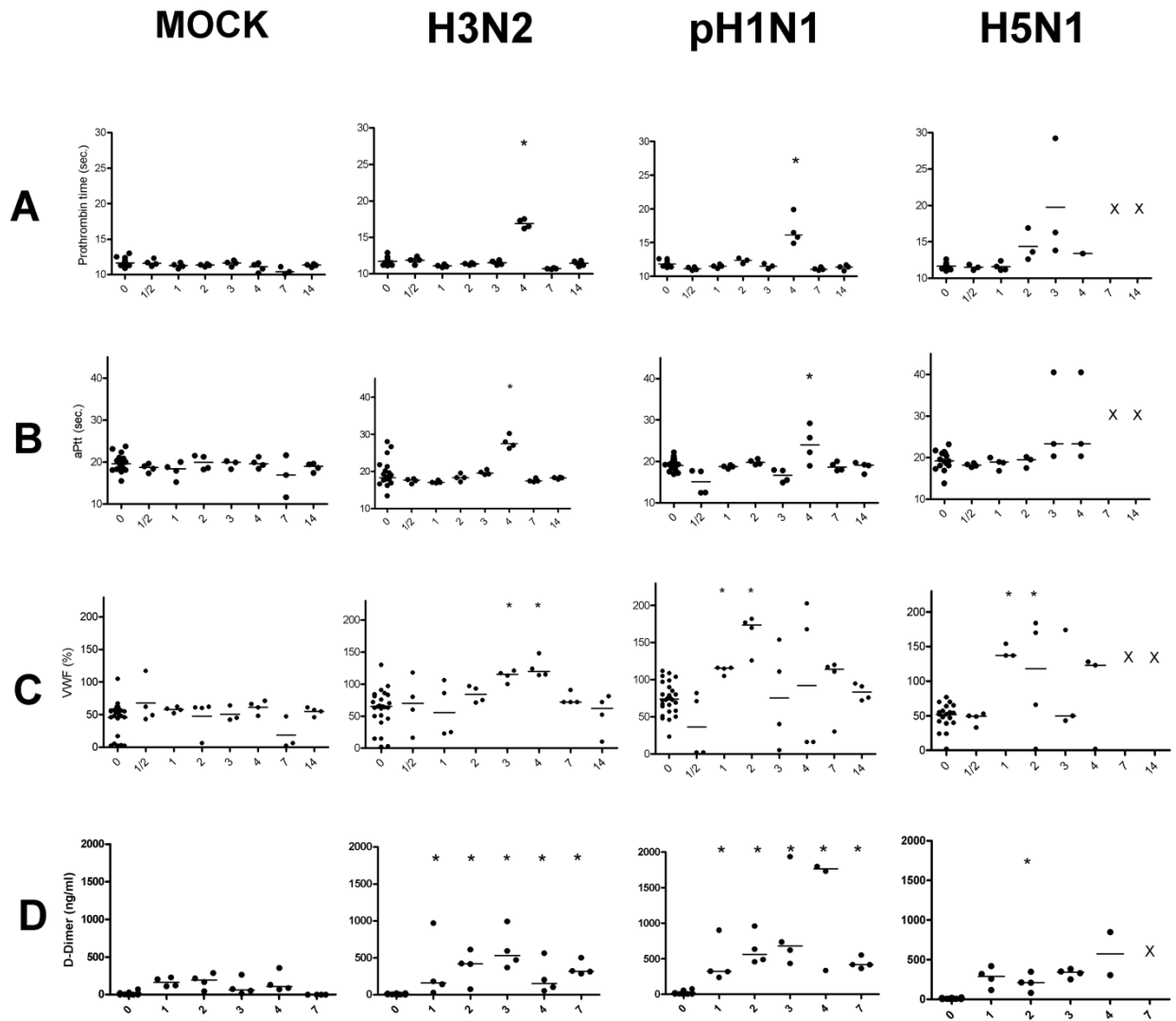


Figure 1. Haemostasis markers during influenza virus infection in ferrets. PT (row A), APTT (row B), VWF activity (row C) and D-dimer levels (row D) in ferrets infected with mock, H3N2-, pH1N1- or H5N1 influenza virus. Asterisk represents a p value < 0.05 in the paired samples ($t = 0$) or compared to the mock infection at the same time point. All influenza variants lead to (transient) increases in PT and APTT. Differences were especially observed on day 4 post infection For PT 18 and for APTT 22 out of 208 samples could not be tested due to technical failure or insufficient plasma volumes. VWF increase is seen in all three influenza virus groups, especially early after infection in pH1N1 and H5N1 virus infected ferrets with statistically significant results in the earliest time points after infection. D-Dimer levels were raised in all 3 influenza groups with the highest levels seen in the pH1N1 virus infected ferrets. X represents no data available since for H5N1 on day 7 and 14 no ferrets were alive.

Increased Von Willebrand factor activity during influenza virus infection in ferrets suggests endothelial cell activation

To study endothelial cell activation Von Willebrand Factor activity (VWF) was measured. Figure 1 (row C) summarizes the results indicating that, compared to mock infection, VWF activity tends to early increase in all three influenza virus infected groups. H3N2 virus infected ferrets showed increased VWF activity from 2 dpi onward. Significant differences were observed at 2, 3 and 4 dpi compared with mock infected ferrets on the same time points (2, 3 & 4 dpi, $p = 0.028$). Compared to all day 0 samples, drawn before inoculation, Mann Whitney U testing shows significant results for 3 and 4 dpi (3 dpi, $p = 0.004$ and 4 dpi, $p = 0.003$). For the pH1N1 virus infected group mean VWF activity differed significantly at 1 and 2 dpi compared to all pre-inoculation samples (1 dpi, $p = 0.0025$, 2 dpi, $p = 0.001$). At these time points, VWF activity was also significantly higher compared to the pre-inoculation samples from the same ferrets in paired testing ($p = 0.03$). HPAI-H5N1 virus infected animals showed trends of increased VWF activity early after infection with highest levels seen at 1 ($p < 0.05$) and 2 dpi ($p < 0.05$).

Increased D-dimer levels during influenza virus infection in ferrets confirms a procoagulant state

D-dimer levels, fibrin degradation products that are markers of both fibrinolysis and coagulation, were quantified and results are listed in row D of Figure 1. Control ferrets had relatively low D-dimer levels with a slight increase the first days after inoculation and returning to normal values at 7 dpi. This increase is most likely associated with the minor inflammation seen after inoculation with the mock cell suspension. After infection, D-dimer levels increased in all infected animals with the highest levels in the H1N1 virus infected animals (Figure 1). D-dimer levels were significantly higher in both the H3N2 and pH1N1 virus infected ferrets at all time points (H3N2 $p = 0.028$; pH1N1 $p = 0.028$) compared to the mock infected group and to the pre-inoculation samples of the same animals (H3N2 $p = 0.005$; pH1N1 $p = 0.003$). D-dimer levels remained higher, compared to mock, until 7 dpi (H3N2 $p = 0.028$ pH1N1 $p = 0.028$). HPAI-H5N1 virus infected animals showed significant increases compared to the pre-inoculation samples ($p = 0.005$) on 2 dpi compared to mock infected ferrets.

Plasma thrombin-antithrombin complexes are especially increased after infection with highly pathogenic avian influenza H5N1 virus

To further analyze activation of coagulation all ferrets were tested for plasma thrombin-antithrombin (TAT) complexes (Figure 2). Highest TAT levels were seen in HPAI-H5N1 virus infected ferrets with a trend of increased TAT generation. To analyze the total TAT formation and compare to D-dimer formation during the course of infection we combined all data from ½ to 4 dpi of each group. This resulted in increased TAT levels for both H1N1 and HPAI-H5N1 virus

infected groups ($p < 0.05$) and an increase in D-dimer formation during all three influenza virus infections (panel E & F Figure 2).

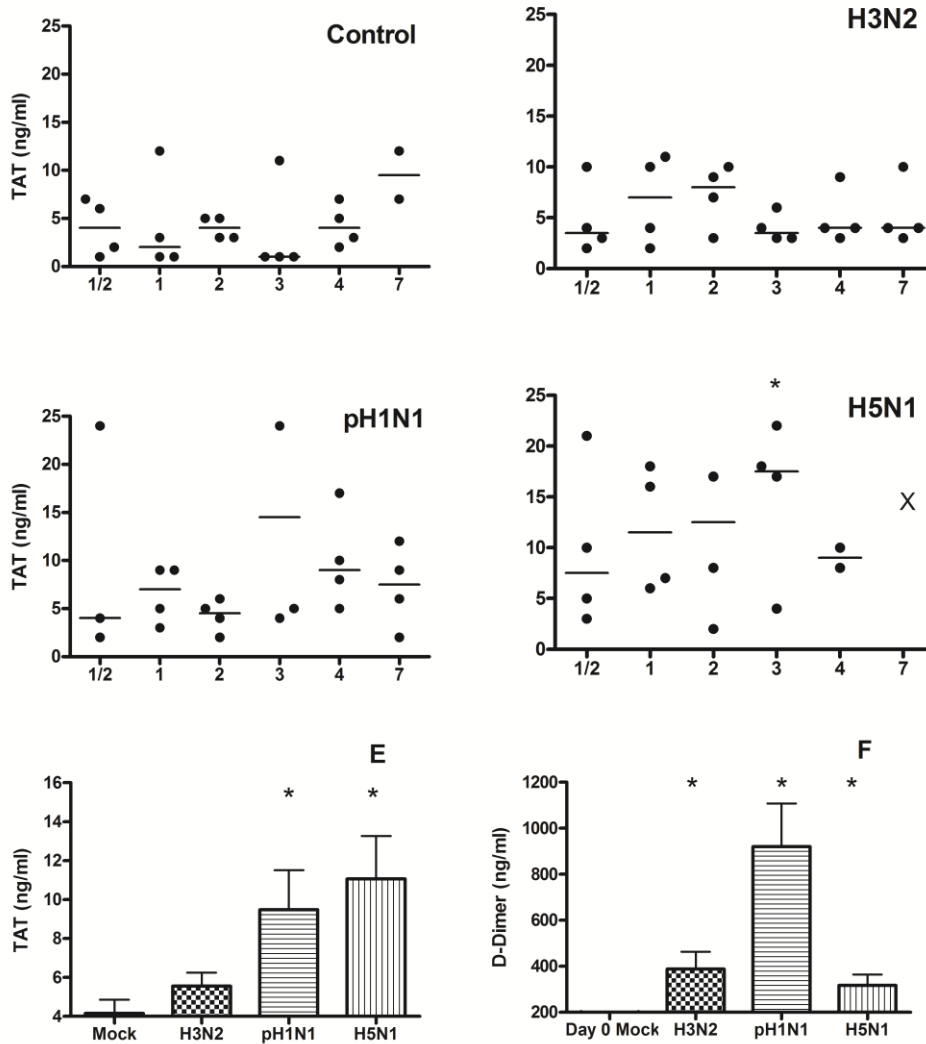


Figure 2. Thrombin-antithrombin complexes in ferrets infected with mock (A), H3N2 (B)-, pH1N (C)- or H5N1(D) influenza virus. Bar represents median in scatterdot. Asterisk represents a p value < 0.05 in the paired samples ($t = 0$) or compared to the mock infection. E shows mean TAT levels during the first episode of infection (day 1/2 to 4) F shows mean D-dimer levels during the first episode of infection (day 1/2 to 4). Samples drawn before infection could not be analyzed due to exogenous TAT formation during venapuncture.

Fibrin tissue staining suggests activation of coagulation at tissue level

The Lendrum staining on alveolar lung tissues showed predominantly the presence of fibrin deposition (orange-red color) in many capillaries in the alveolar walls in the HPAI-H5N1 virus infected group (Figure 3). Intermediate numbers of capillaries stained positive in the H3N2 virus infected group, a few capillaries of the pH1N1 virus infected group and in none in a negative control sample from an uninfected ferret. However, the differences did not reach statistical significance when compared to the mock infected group. The mock infected group inoculated with uninfected cell derived material did show minor signs of inflammation which were the result of intra tracheal inoculation. This resulted in an intermediate numbers of capillaries positive for fibrin staining. In the slides stained for fibrin, there is no or very little presence of fibrin in the lumen of the bronchial submucosal glands with no significant difference between the virus groups. Only in few pH1N1 and H5N1 infected animals in rare lumina of bronchial submucosal glands there was little staining of fibrin, despite the differences in inflammation within the glands between the viruses. The staining pattern in the capillaries surrounding the bronchi is similar as that in the lung parenchyma.

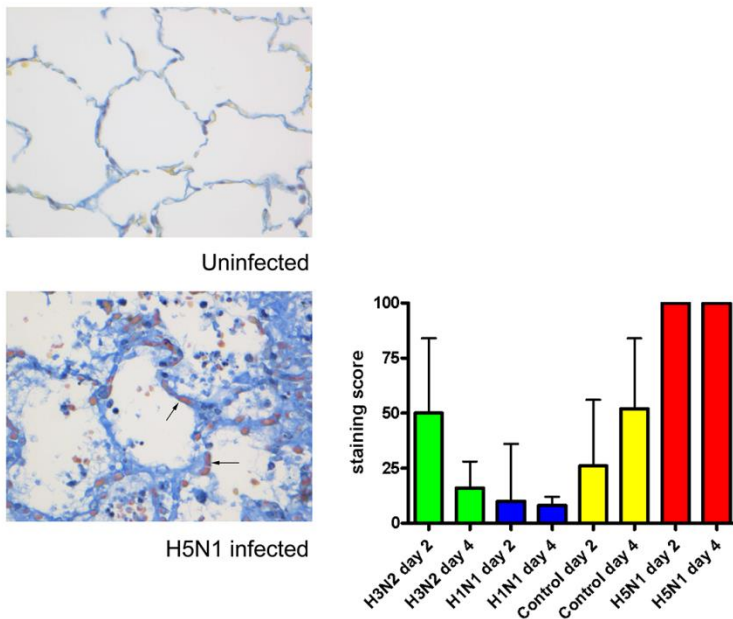


Figure 3. Fibrin (Lendrum) staining of ferret lung tissue. Lendrum staining expressing fibrin (red) in lung tissue of a control ferret or 4 days after inoculation of different influenza viruses. No staining in a non-infected ferret (A), occasional intracapillary staining of fibrin in ferrets inoculated with H3N2 (B) and pH1N1 (C), and multifocal intracapillary staining in ferrets inoculated with H5N1 (D). Panel E shows the results of a semi-quantitative scoring of fibrin deposition obtained by examining 25 images per slide.

Comparison of coagulation parameters with virological and disease severity data.

In HPAI-H5N1- and pH1N1 virus infected animals VWF activity increased in the first two days after infection, coinciding with peak virus titres. D-dimer levels increased during the first days after infection to peak at 3 and 4 dpi, when virus titres started to significantly decrease. In these animals, highest levels in clotting times were seen at 4 dpi when a peak in relative lung weights was also observed. There was a significant correlation between multiple parameters in all three influenza groups (summarized in Table 3). Correlation analysis revealed positive correlation between PT values and AUC of the virus titres for the H3N2 virus ($R = 0.8$, $p < 0.01$) and pH1N1 virus ($R = 0.7$, $p < 0.01$). D-dimer levels significantly positively correlated with virus titre AUC and body weight decrease for the pH1N1 virus infected group. If we combine all data and thereby generate a dataset from influenza A virus infected ferrets, significant positive correlations can be seen between many of the virological and clinical parameters compared to the coagulation parameters. All significant R values are listed in Table 4 with those of most interest being body weight decrease with VWF, PT, APTT and D-dimer levels. Virus titre AUC's correlated significantly with PT, APTT and D-dimer levels.

<i>Correlation</i>	<i>H3N2</i>	<i>pH1N1</i>	<i>H5N1</i>	<i>H1N1+H5N1</i>	Influenza A
PT -Titre total [#]	NS	-0.6 (-0.9—0.1) *	NS	-0.5 (-0.75- -0.1) *	NS
PT -AUC total [#]	0.8 (0.4-0.9) ***	0.7 (0.3-0.9) **	NS	0.4 (0.1-0.7) *	0.4 (0.2-0.7)**
PT -Body weight	NS	0.8 (0.4-0.9)**	NS	0.5 (0.1-0.7)*	0.5 (0.2-0.7) **
PT -Lung weight	NS	0.6 (0.05-0.9) *	NS	NS	0.4 (0.05-0.6) *
APTT -Titre total [#]	-0.5 (-0.8 - -0.1)	NS	NS	NS	NS
	*				
APTT -AUC total [#]	0.8 (0.6-0.9) ***	NS	NS	NS	0.3 (0.05-0.6) *
APTT -Body weight	NS	0.6 (0.2-0.9) **	NS	0.5 (0.1-0.7) **	0.4 (0.2-0.6) **
APTT -Lung weight	NS	NS	NS	NS	0.3 (0.1-0.6) *
VWF-Titre total [#]	-0.6 (-0.8-0.1) *	NS	NS	NS	NS
VWF-AUC total [#]	0.7 (0.4-0.9)**	NS	NS	NS	NS
VWF-Body weight	NS	NS	NS	NS	0.4 (0.1-0.6) *
VWF-Lung weight	NS	NS	NS	NS	NS
D-dimer -Titre total [#]	NS	NS	NS	NS	NS
D-dimer -AUC total [#]	NS	0.6 (0.2-0.8)*	NS	0.5 (0.1-0.7) *	0.4 (0.2-0.6)**
D-dimer -Body weight	NS	0.7 (0.2-0.9) **	NS	0.5 (0.2-0.7)**	0.5 (0.2-0.7) ***
D-dimer -Lung weight	NS	NS	NS	NS	NS
TAT -Titre total [#]	NS	NS	NS	NS	0.3 (0.1-0.6) *
TAT -AUC total [#]	NS	NS	NS	NS	NS
TAT -Body weight	NS	NS	0.6 (0.2-0.9) *	NS	NS
TAT -Lung weight	NS	NS	NS	0.5 (0.1-0.7)**	0.3 (0.01-0.5) *

Table 4. Correlation between virological parameters and markers of haemostasis.

Virological parameters are listed in table 2. For each influenza virus group coagulation values were correlated to virological and clinical parameters. This was also done for the complete influenza A group (H3N2 + pH1N1 + H5N1) and for the combination of pH1N1 and H5N1 because these two viruses are able to infect the complete respiratory tract instead of only the upper respiratory tract which is the case for H3N2. Pearson correlation coefficients are given if the values were statistically significant. * $p < 0.05$ ** $p < 0.01$ *** $P < 0.001$ if not significant NS is listed in the table.

Discussion

The present study demonstrates, for the first time, procoagulant effects at the circulatory and tissue level in a ferret influenza model, largely proportional to the severity of influenza virus infection. These findings are in line with earlier epidemiological, clinical, animal and *in vitro* data (69;84-86;455;460;466;467). Ferrets have been shown to be an adequate model to study the coagulation cascade (468-470) with PT and APTT normal values varying from 11.6-12.7 and 18.9-22.3 seconds respectively. This is comparable to our 104 pre-inoculation ferret samples (PT 11.7 (+/- 0.1) and APTT 19.8 (+/- 2.2)) (469).

Like in humans, highly pathogenic avian influenza virus infection causes severe disease in ferrets, which may include bleeding complications and multi-organ failure (471;472). In our experiments, HPAI-H5N1 virus inoculated ferrets showed severe disease, which in some cases resulted in spontaneous death. Analysis of the coagulation cascade in these animals confirmed the severity of infection with prolongation of global coagulation assays and signs of activated endothelium. PT and APTT values increased already from 2 dpi onward with individual ferrets showing an increase up to 20 seconds. This observation is suggestive for consumptive coagulopathy which is strengthened by the high levels of fibrin deposition in the lung capillaries. Consumptive coagulopathy could be the result of extreme activation of coagulation, for instance due to increased tissue factor production as is seen in other (severe) viral diseases as Ebola haemorrhagic fever. The exact role for consumptive coagulopathy in highly pathogenic H5N1 infection warrants further research, but hypothetically the excess of coagulation activity could lead to microthrombosis in the pulmonary alveoli leading to respiratory distress or even multi organ failure (47). The procoagulant changes were seen both at the tissue level and in the circulation, suggested by the TAT increase. The statistically significant increase in D-dimer levels confirms this procoagulant state. However, D-dimer levels were lower in HPAI-H5N1 virus inoculated ferrets compared to ferrets infected with H3N2 virus and especially compared to the ferrets infected with pH1N1 virus. A possible explanation for this phenomenon could be the inhibition of fibrinolysis by high levels of plasminogen-activator type 1 activity (PAI-1) during H5N1 virus infection. Unfortunately we could not test PAI-1 activity in ferret plasma with the currently available human PAI-1 activity assays. Since plasminogen is proven to play an important role in influenza pathogenesis further exploring the biology, activation and inhibition of plasminogen in influenza infection would be of great interest (473).

The second virus we used in our experiments was pH1N1. Although less severe compared to HPAI-H5N1 virus infected ferrets, pH1N1 virus infection caused severe pneumonia with lung damage in ferrets. While ferrets infected with pH1N1 virus showed remarkably high levels of D-dimer, tissue fibrin deposition was not as prominent as seen in HPAI-H5N1 virus infected ferrets. Activated coagulation in other organs than the respiratory tract or a systemic activation of coagulation could explain this phenomenon. These severe procoagulant changes in the circulation could be the result of a specific immune activation during pH1N1 virus infection. A

possible explanation can be found in the work of Monsalvo et al. who showed an excessive amount of pathogenic immune complexes, which are known to have systemic procoagulant effects, in fatal pH1N1 cases (474;475). Furthermore, TAT levels significantly increased in the first 4 days after infection and at 4 dpi there was a remarkable prolongation of PT and APTT values up to 4 seconds. The very 'sudden' increase of clotting times at 4 dpi is suggestive for a consumptive coagulopathy, possibly similar to what was seen in DIC due to HPAI-H5N1 virus infection and bacterial sepsis (476). Clotting times had normalized at 7dpi, however, indicating that in contrast to bacterial sepsis, the consumptive coagulopathy is transient and less severe. The 'sudden' onset of clotting time prolongation may be of interest to evaluate specific coagulation factor changes during influenza infection.

To evaluate the influence of a more 'moderate' influenza virus infection, seasonal H3N2 virus was also included in the experiments. Although this influenza virus in general causes 'moderate' disease in humans and ferrets, it did cause significant procoagulant changes in the model with hemostatic alteration comparable to those of pH1N1 virus infected ferrets. However, TAT levels did not increase suggesting a more moderate procoagulant state compared to H1N1- and H5N1 virus infected animals.

Since the ageing human population is prone to both an increase in cardiovascular disease and to complications during and after infection with seasonal and avian influenza viruses (477;478), further exploration of the interplay between influenza and haemostasis would be of great interest. Most of the associations found in Table 3 show positive correlations between coagulation parameters and markers of inflammation (body weight decrease and relative lung weight increase). This comes as no surprise since the bidirectional cross-talk between coagulation and inflammation has been studied very well, whereby inflammation in general evokes a procoagulant response (479-481). The specific disturbances in the tightly regulated balance between clotting, anti-coagulation and inflammation could be a target for novel intervention strategies in influenza. Following our observational study, an intervention model could further evaluate the role of coagulation in influenza virus pathogenesis and the potential processes for targeted intervention, for example by targeting protease receptor type-2 (PAR-2) activation in influenza pathogenesis. PAR-2 is an important receptor in both inflammation and coagulation, and recently described to have a major role in the damage seen after the inflammatory response during influenza virus infection (482;483). While statins may also be interesting candidates for future studies. Statins may counteract specific inflammatory responses such as seen after acute coronary syndrome, and thereby may decrease mortality when given to influenza patients. Studying the influence of statin treatment on the procoagulant changes during influenza virus infection and the role these changes have in the postulated increased risk of myocardial infarction would be of great interest (484-486).

Collectively the data generated by our study will pave the way for further exploration of novel treatment and intervention strategies for influenza and its complications. Furthermore, based

on the correlation between the viral infection - and coagulation parameters in this experiment, coagulation tests could serve as valuable biomarkers predicting disease severity. The ferret model likely offers the best opportunity to explore these options in a preclinical setting optionally also linked to host genetics since ferrets represent an outbred population.

CHAPTER 4

ECOLOGY & PREVENTION: “Newly identified risk factors and tools of prevention”

Consists of:

- 4.1** The association between hantavirus infection and selenium deficiency in mainland China
- 4.2** Safety and immunogenicity of an MVA-based influenza A/H5N1 vaccine. A randomized phase I/IIa clinical trial.

CHAPTER 4.1

The association between hantavirus infection and selenium deficiency in mainland China

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Abstract

“Haemorrhagic fever with renal syndrome (HFRS) caused by hantaviruses and transmitted by rodents is a significant public health problem in China, and, based on the analysis of high endemic HFRS areas, seems to occur more frequently in selenium-deficient regions. To study the role of selenium concentration in HFRS incidence we used a multidisciplinary approach combining ecological analysis with preliminary experimental data. The incidence of HFRS in humans was about six times higher in severe selenium-deficient and double in moderate deficient areas compared to non-deficient areas. This association became statistically stronger after correction for other significant environment-related factors (low elevation, few grasslands, or an abundance of forests) and was independent of geographical scale by separate analyses for different climate regions. A case-control study of HFRS patients admitted to the hospital revealed increased activity and plasma levels of selenium binding proteins while selenium supplementation in vitro decreased viral replication in an endothelial cell model after infection with a low multiplicity of infection (MOI). Viral replication with a higher MOI was not affected by selenium supplementation. Our findings provide a valuable clue that selenium deficiency contributes to an increased prevalence of hantaviruses infections in both humans and rodents. However, future studies should further examine the exact mechanism behind this observation before selenium supplementation in deficient areas could be implemented for HFRS prevention.”

Introduction

Haemorrhagic fever with renal syndrome (HFRS) is a zoonotic disease with a severe clinical presentation characterized by, as the name suggests, fever and renal failure potentially complicated by haemorrhage and shock (297). The disease is the result of infection with an Old-World pathogenic hantavirus, which are negative stranded RNA viruses from the *Bunyaviridae* family (307). Pathogenic hantaviruses are shed in the excreta of their reservoir rodents and in most cases each hantavirus is associated with a specific rodent host (296). In mainland China HFRS is either caused by infection with the Hantaan virus (HTNV) or the Seoul virus (SEOV) carried by the striped field mice (*Apodemus agrarius*) and Norway rats (*Rattus rattus*) respectively (116;487-489). HFRS is a significant public health problem in mainland China with an annualized average incidence reported up to 120 per 100,000 persons per year for the high endemic parts of the country (489). Case fatality rates (CFR) range from 0.1% to 15%, depending on the causative hantavirus and specific characteristics of the infected individual (54). To increase insight in HFRS epidemiology in China we previously identified hantavirus counties with the highest HFRS incidence per year in China and defined them as hantavirus 'hotspots' (489). Further analysis of these hotspots revealed that most of them are categorized as being a 'selenium-deficient' area. This made us decide to further explore the association between hantavirus infection and selenium deficiency.

Selenium is an essential micronutrient for many life forms, including humans, and has been suggested to play a role in multiple physiological and pathological processes (490;491). In contrast to many other micronutrients, the intake of selenium varies worldwide, ranging from decreased or even a severe deficient dietary intake to an elevated selenium intake, while even toxic concentrations in crops and feed have been reported in literature (492-494). Low intake of selenium, subsequently leading to decreased selenium levels in the circulation, might directly lead to diseases such as endemic cardiomyopathy (Keshan disease) or deformative osteoarthritis (Kashin-Beck disease) which is a relatively common disease in China (495-497). Furthermore, multiple studies suggest selenium levels to influence the occurrence and outcome of multiple other diseases including cancer and heart disease. Most important for this study is the fact that the association between selenium levels and disease outcome is also seen in diseases of infectious origin (498;499). For instance, the incidence, virulence and progression of viral infections like human immunodeficiency virus (HIV), coxsackie virus, and influenza virus infections are influenced by selenium deficiency (500-503). The exact role of selenium concentration in these infections is not totally understood. However, experimental studies show selenium to play multiple roles in the immune response to infectious diseases. It has been shown that selenoproteins are essential for activated T-cell function and it seems they are involved in the modulation of inflammatory responses, for instance by inducing the interferon response (504-507). Selenium deficiency affects T-cell immunity by suppressing T-cell proliferation and causing defects in T-cell-dependent antibody responses (508).

To explore the relation between HFRS incidence and selenium concentration in China, we decided to use a multi-disciplinary translational approach combining ecological, clinical and *in vitro* experiments. The aims of our study are 1) to examine to which extent human HFRS incidence and hantavirus infection rates of human and rodent reservoirs are associated with measures of selenium deficiency, controlling for other potentially confounding environmental factors, and 2) to explore where in the chain of the occurrence of a hantavirus infection, from rodent reservoir to human case, selenium concentration might have its influence. By studying levels of selenium binding proteins in acute HFRS patients (glutathione peroxidase (GPx3)) and the *in vitro* effects of selenium supplementation in a well-established puumala hantavirus (PUUV) endothelial cell model (HUVEC).

Results

Ecological analysis

During 2005-2010, a total of 74,118 HFRS cases, including 914 deaths, were reported in 1,960 of the 2,922 counties in mainland China. The average annual HFRS incidence varied greatly over counties, ranging from 0 to 54 per 100,000 person-years, with a median of 0.1 per 100,000 person-years. Areas were divided by selenium concentration in crops and feed in to selenium non-deficient (≥ 0.06 ppm), moderate-deficient (0.03 - 0.05 ppm) and severe deficient (≤ 0.02 ppm) areas. The moderate- and severe selenium-deficient areas covered 37.3% and 31.8% of the country, respectively (Figure 1). The average annual HFRS incidence in severe selenium-deficient areas (2.27 per 100,000 person-years) was almost 3 times higher than in moderate deficient areas (0.83 per 100,000 person-years), which on its turn was double that in non-deficient areas (0.40 per 100,000 person-years) (Table 1). Both of the hotspots of highest incidence of HFRS were located on the selenium-deficient belt of China: one in the eastern areas of northeastern China, the other in the central areas of mainland China (Figure 1).

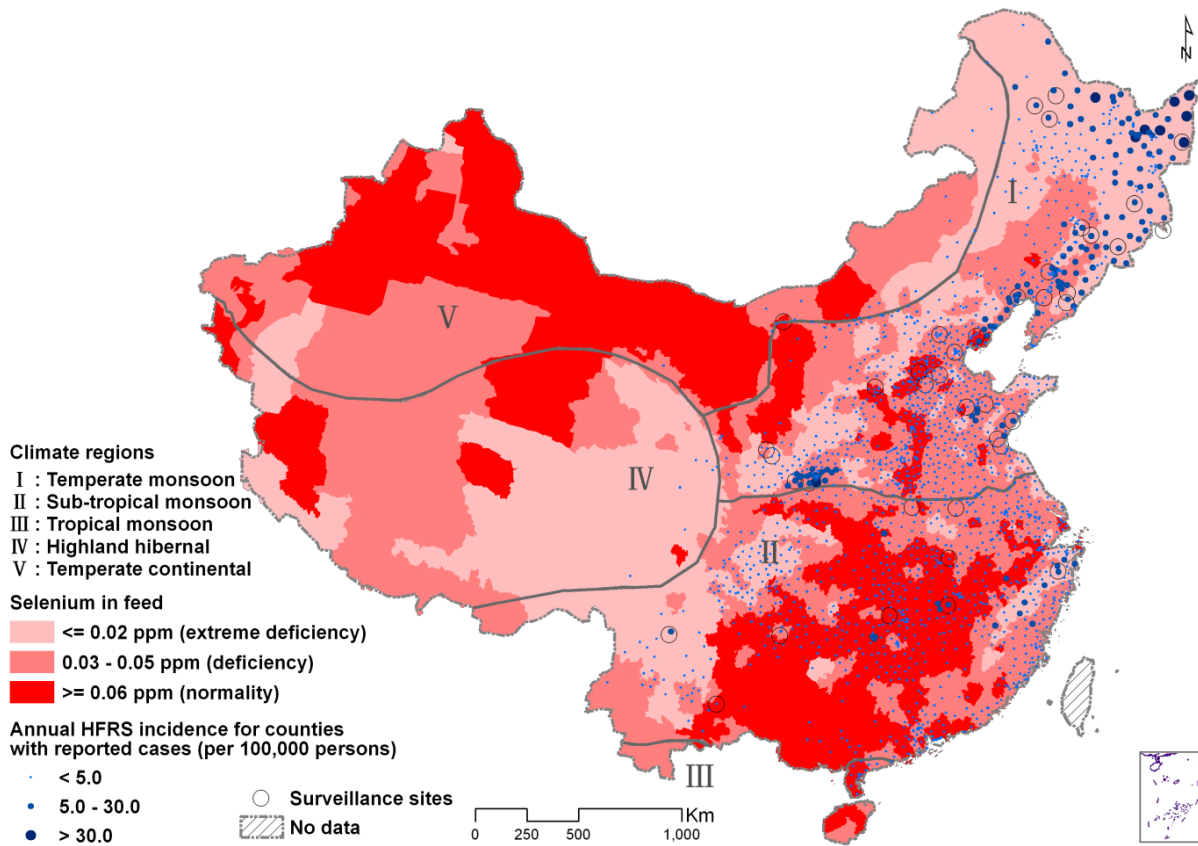


Figure 1. Geographic distribution of hemorrhagic fever with renal syndrome (HFRS) incidence in relation to selenium content of crops and feed in mainland China. *The background of the map with color gradient presents the selenium content of crops and feed, and the dots with size and color gradient display the average annual incidence of HFRS from 2005 to 2010. Areas without dots do not have reported cases of HFRS. The thick grey lines are boundaries of 5 climate regions of China. The thin black circles indicate the 40 surveillance sites for hantaviruses infection in rodent hosts (used in Figure 2). Ppm is parts per million.*

Variables (unit) ^a	Average yearly incidence (95% CI, per 100,000 person-years)	Univariate analysis		Multivariate analysis	
		Crude IRR (95% CI)	P-value	Adjusted IRR (95% CI)	P-value
Selenium content (categorical, ppm)					
>= 0.06 (non-deficient areas)	0.40 (0.32 - 0.48)	1	-	1	-
0.03 - 0.05 (moderate deficient areas)	0.83 (0.70 - 0.95)	2.07 (1.73 - 2.46)	< 0.001	2.43 (2.04 - 2.88)	< 0.001
<= 0.02 (severe deficient areas)	2.27 (1.85 - 2.70)	5.68 (4.64 - 6.95)	< 0.001	7.77 (6.29 - 9.61)	< 0.001
Elevation (categorical, 1000 m)					
< 0.4	1.33 (1.14 - 1.52)				
0.4 -	1.37 (1.05 - 1.70)				
0.8 -	0.58 (0.35 - 0.81)				
1.6 -	0.17 (0.00 - 0.33)				
> 3.2	0.08 (0.00 - 0.16)				
Elevation (continuous, 1000 m)		0.46 (0.41 - 0.50)	< 0.001	0.40 (0.35 - 0.46)	< 0.001
Croplands (categorical, %)					
< 20	0.98 (0.76 - 1.20)	1	-	1	-
20 -	1.03 (0.84 - 1.23)	1.04 (0.86 - 1.26)	0.659	1.14 (0.93 - 1.39)	0.200
> 50	1.13 (0.91 - 1.35)	1.14 (0.94 - 1.38)	0.185	1.03 (0.84 - 1.27)	0.766
Forests (categorical, %)					
< 5	0.90 (0.72 - 1.09)	1	-	1	-
5 -	1.01 (0.81 - 1.21)	1.11 (4.62 - 7.11)	0.285	1.53 (1.26 - 1.87)	< 0.001
> 40	1.21 (0.98 - 1.45)	1.32 (1.42 - 2.27)	0.004	1.53 (1.26 - 1.86)	< 0.001
Grasslands (categorical, %)					
< 2	1.20 (1.02 - 1.38)	1	-	1	-
2 -	1.41 (1.13 - 1.70)	1.18 (0.98 - 1.41)	0.082	0.92 (0.77 - 1.10)	0.365
> 20	0.44 (0.32 - 0.55)	0.38 (0.31 - 0.45)	<	0.58 (0.45 - 0.74)	< 0.001

			0.001		
Population density (categorical, 1000 persons per km²)					
< 0.2	1.38 (1.10 - 1.65)				
0.2 -	0.98 (0.82 - 1.14)				
> 0.5	0.73 (0.61 - 0.84)				
Population density (continuous, 1000 persons per km²)		0.97 (0.95 - 0.99)	0.001	1.00 (0.98 - 1.02)	0.728
GDP (categorical, 10 million Yuan per km²)					
< 0.08	1.22 (0.94 - 1.50)				
0.08 -	1.07 (0.90 - 1.25)				
> 0.4	0.84 (0.70 - 0.96)				
GDP (continuous, 10 million Yuan per km²)		0.91 (0.88 - 0.93)	< 0.001	0.93 (0.90 - 0.96)	< 0.001

Table 1. The association between haemorrhagic fever with renal syndrome (HFRS) incidence and selenium content of crops and feed by Poisson regression, using data from 2005 to 2010 in mainland China, both univariately and corrected for other influencing factors in multivariate analysis. IRR is incidence rate ratio. CI is confidence interval. For all continuous variables, we also report categorical results to allow inspection of the data and to assess whether the continuous assumption was justified. These categorical variables were not considered in the Poisson regression.

Variables (unit)	Region I		Region II & Region III	
	Adjusted IRR (95% CI)	P-value	Adjusted IRR (95% CI)	P-value
Selenium content of crops and feed (categorical, ppm)				
>= 0.06 (non-deficient areas)	1		1	
0.03 - 0.05 (moderate deficient areas)	1.70 (1.27 - 2.27)	< 0.001	0.85 (0.67 - 1.08)	0.181
<= 0.02 (severe deficient areas)	3.45 (2.49 - 4.79)	< 0.001	2.80 (1.92 - 4.09)	< 0.001

Table 2. The association between HFRS incidence and selenium content by multivariate Poisson regression in different climate regions, corrected for other influencing factors (not shown). Region I = temperate monsoon climate; Region II = sub-tropical monsoon climate; Region III = tropical monsoon climate. Region II and Region III were combined due to the small area of Region III. In Region IV (highland hibernal climate) and Region V (temperate continental climate), almost all cases were located in severe selenium-deficient and moderate selenium-deficient areas, except for 2 cases locating in non-deficient areas in Region V, not allowing statistical analysis due to the low numbers. IRR is incidence rate ratio. CI is confidence interval.

Univariate Poisson regression analyses revealed that HFRS incidence was significantly associated with selenium content of crops and feed, average elevation, proportion of areas of forests and grasslands, population density, and GDP. Multivariate analysis showed that the adjusted IRR (7.77) in severe selenium-deficient areas was even higher than the crude IRR (5.68) in comparison with non-deficient areas (Table 1), while elevation, proportion of areas of forests and grasslands, and GDP remained significantly associated with HFRS incidence.

Although selenium content was disproportionately spread over the five climate regions of China (Figure 1), similar associations between HFRS incidence and selenium content could be demonstrated within these regions (Table 2). The regions with many severe selenium-deficient areas had the highest IRR in Region I, and in Regions II and III combined. In Region IV, only 58 HFRS cases were reported, but all of them came from severe selenium-deficient areas. Out of the 131 reported HFRS cases in Region V, 28, 103, and 2 cases were located in severe selenium-deficient areas, moderate deficient areas, and non-deficient areas, respectively (Figure 1), and the corresponding annual incidences were 0.073, 0.152, and 0.003 per 100,000 human-years, respectively. Due to the low numbers, statistical analyses were not conducted for Regions IV and V.

To verify the association between selenium deficiency in crops and feed and the percentage of infected reservoir rodents, the hantavirus infection rate of rodent hosts in relation to selenium content of crops and feed was investigated for 40 surveillance sites through China (see the circles in Figure 1 for their locations). Both HFRS incidence in humans (Figure 2A) and hantavirus infections in rodents (Figure 2B) were negatively correlated with selenium content of crops and feed. The nonparametric equality-of-medians tests indicated that a significant difference on both HFRS incidence in humans ($\chi^2 = 15.6$, $P < 0.001$) and hantaviruses infection rates in rodents ($\chi^2 = 6.8$, $P = 0.034$) were found among these three areas.

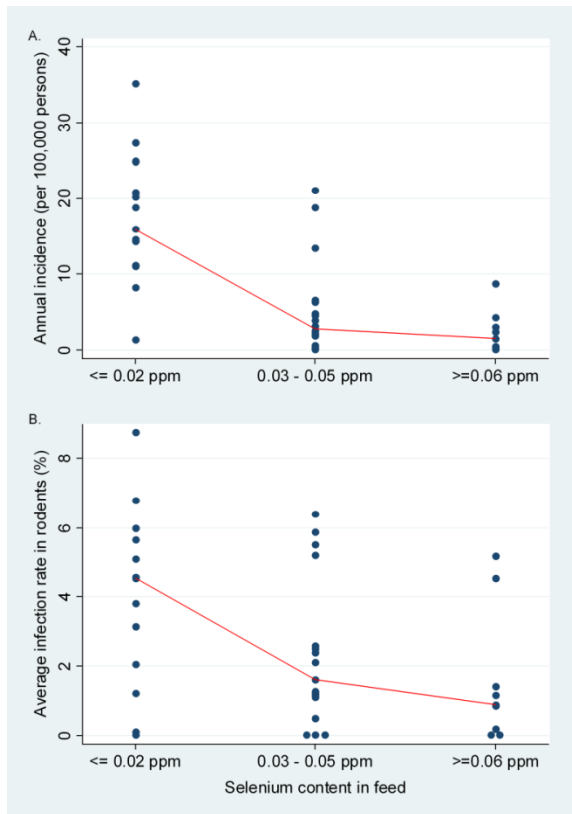


Figure 2. Correlation between HFRS incidences in humans, hantaviruses infections in rodents and selenium content.

The rodent data were from 40 surveillance sites in mainland China during the years 2005 - 2008. The points reflect (A) average incidences of HFRS in human for the counties each surveillance site and (B) annual average hantaviruses infections in rodents for each surveillance site, and the lines represent the median bands. Selenium content of crops and feed ≤ 0.02 ppm represents severe selenium-deficient areas; 0.03 - 0.05 ppm represents moderate selenium-deficient areas; ≥ 0.06 ppm represents non-deficient areas.

Clinical analysis

Glutathione peroxidase (GPx3) concentrations and GPx activity in acute HFRS patients

To study whether selenium levels differed between HFRS cases and healthy controls a case-control study was executed which assessed the glutathione peroxidase 3 (GPx3) concentrations and glutathione peroxidase (GPx) enzyme activity, the major selenium binding proteins in humans, in acute HFRS patients and healthy controls. In total, 77 acute HFRS cases were included for GPx3 concentration measurement of which 61 (79.2%) were male. Concentration levels were compared to GPx3 concentrations measured in 72 healthy controls of which 36 were male (50%). Age did not differ significantly between the two groups ($p = 0.6$). GPx3 concentrations in ng/ml are shown in Figure 3. Acute male HFRS patients showed mean GPx3 levels of 0.56 (0.28-0.84 ng/ml), while male healthy controls showed statistically significant lowered GPx3 levels of 0.28 (0.19-0.37) ($p < 0.001$). In female HFRS patients, a similar trend was observed showing statistically significant increased levels of GPx3 in HFRS patients compared to female healthy controls: 0.56 (0.23-0.89 ng/ml) versus 0.26 (0.19-0.33 ng/ml) ($p = 0.001$).

GPx activity levels were measured in a second cohort of HFRS patients. Samples from 80 acute HFRS cases (87.5% male) and 85 healthy controls (54.1% male) were tested for GPx activity. Again, age did not differ significantly between the two groups ($p = 0.2$). Figure 3 visualizes the increased GPx activity levels in acute male HFRS patients versus controls ($p = 0.001$), while there is no significant difference between cases and controls for female HFRS patients.

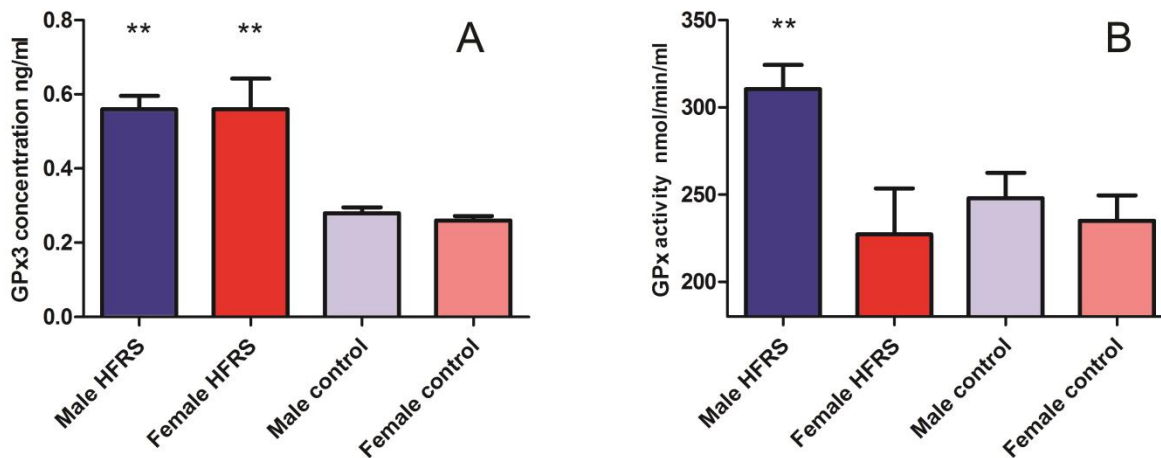


Figure 3. Glutathione peroxidase (GPx3) concentrations (A) and GPx activity (B) in HFRS patients and healthy controls. Asterisks ()** represent a *p* value smaller than 0.01 ($p < 0.01$). GPx3 levels (A) were increased in acute HFRS patients compared to controls. Furthermore, GPx enzyme activity levels (B) in nmol/min/ml were higher in male HFRS patients compared to controls.

In vitro analysis of the influence of selenium on hantavirus replication

To study the influence of selenium concentration on hantavirus replication in a well-established immune competent cell line of human origin we infected Human Umbilical Vein Endothelial Cells (HUVEC) with a low passage virulent isolate of puumala hantavirus (PUUV) with or without selenium supplementation. Figure 4 shows the viral copy numbers in each replicate measured every 24 hours up to 72 hours post infection of HUVEC's with a multiplicity of infection (MOI) of 0.05. Viral copy numbers significantly decreased after selenium supplementation of 200 ng/ml, suggesting a decrease in viral replication. If the number of virus particles of the inoculum increased to MOI 1 or even 3 the decrease in virus replication was no longer significant. This suggests that only at a low percentage of infected cells, after infection with a low MOI, selenium supplementation of 200 ng/ml affects viral replication.

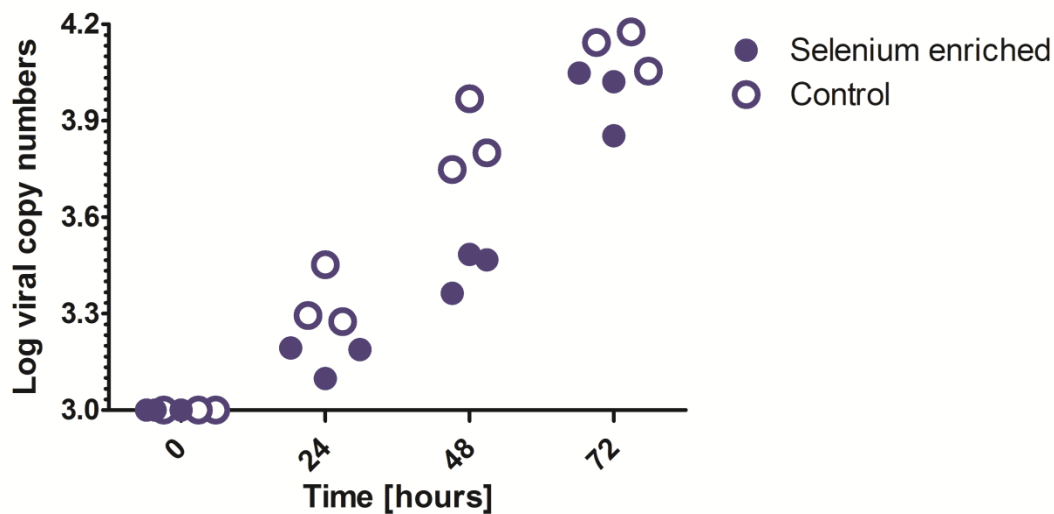


Figure 4. Viral copy numbers with or without the supplementation of selenium to cultured endothelial cells infected with Puumala hantavirus. *Selenium supplementation (200 ng/ml of sodium selenite) to the culture medium leads to decreased viral replication after infection with a multiplicity of infection (MOI) of 0.05. Data was 10-log transformed to reach a normal distribution.*

Discussion

HFRS is one of the most severe viral infectious diseases in mainland China (350;488). Furthermore, China is known to be one of the most severely selenium-deficient countries in the world (509;510), while in multiple aspects selenium deficiency is linked to pathophysiological mechanisms (504;506). By analyzing all reported HFRS cases in mainland China during a 6-year period, we found that the annual HFRS incidence was significantly lower in non-selenium-deficient areas, relative to moderate selenium-deficient and severe selenium-deficient areas.

After adjusting for other influential factors, the incidence of HFRS remained significantly associated with selenium deficiency. In severe selenium-deficient areas, the average HFRS incidence was about 6 times higher than that in non-deficient areas. The association between HFRS incidence and selenium deficiency was present in all 5 climate regions of China. Furthermore, HFRS incidence in humans and hantaviruses infection rates in rodent hosts were negatively correlated with selenium for 40 surveillance sites across the country. The highly heterogeneous distribution of HFRS incidence and the strong variation in selenium content of crops and feed give us a unique opportunity to explore the possible link between HFRS incidence and selenium.

The low incidence of HFRS in southwestern China, which is a commonly selenium-deficient area shows that selenium deficiency could not be the only determinant for the occurrence of HFRS. Other factors influencing the reservoir distribution and rodent exposure should also be

considered for the occurrence of HFRS. In our study, we found low elevation to be another important determinant for HFRS in mainland China, which could be explained by the preference for low elevation by the HNTV reservoir species (489;511). Similarly, the distribution of land use (croplands, forests and grasslands) is associated with agricultural activities, possibly related to rodent densities. Furthermore, population density and GDP reflect the human living conditions and could very well relate to potential exposure of humans to rodents and their excreta (297). All of these determinants, despite selenium deficiency, were unfavorable for HFRS incidence in southwestern China.

The very strong association between HFRS incidence and selenium concentration made us further explore which mechanisms potentially underlie this observation. The mechanisms that drive HFRS incidence are complex and multi-factorial (324;512) but basically include three main segments: 1) reservoir ecology, 2) virus ecology and 3) human factors. Interpretations of earlier studies on selenium and infectious disease suggest a potential role of each of these segments. By executing the first preliminary experiments on the potential mechanisms between HFRS and selenium, we tried to estimate how selenium concentrations most likely have their effect. At first we looked at human factors in the relation between selenium and HFRS by performing a case-control study in HFRS cases and comparing the plasma concentration and activity of the major selenium binding proteins GPx and GPx-3 (513). We thereby hypothesized that deficiency in selenium could either increase the hosts' susceptibility to hantavirus infection, leading to more infections, or increase the host response to the hantavirus infection, which would lead to a more severe disease and thus an increase of reported HFRS cases. However, HFRS patients showed an increase in GPx3 levels, and also GPx activity was significantly higher in acute HFRS male patients. This observed pattern seems contradictory to the hypothesis that selenium deficiency affects the human factor in the occurrence of a HFRS case, especially since other studies describe a decrease in GPx3 activity during viral infection and sepsis (514;515), and suggests that the possible mechanism has its effect on the reservoir rodent or the virus.

Our preliminary *in vitro* data show that selenium supplementation decrease viral replication in immune competent cells after infection with a (very) low infectious dose. However, increasing the infectious dose eliminated this effect, suggesting a role for selenium only in low viral load infections *in vitro* and thereby a (weak) immune stimulant effect of selenium supplementation. This effect could be bigger *in vivo* since earlier observed effects of selenium supplementation on natural killer- and T cell activity do not play a role in the *in vitro* cell model. This suggests that the mechanism behind our observation does not lie in the human host but might concern a prolonged or increased viral load in the reservoir, which potentially leads to prolonged and increased virus shedding. Furthermore, a direct mutating effect of selenium on the virus, resulting in changes in the viral fitness, cannot be excluded. This is an observation that has also been described in influenza virus studies (516;517). Unfortunately with our current assays and the samples available we were not able to measure selenium concentrations

in the reservoir rodents. To fully address these questions, further experiments should focus on selenium levels in the reservoir species, for instance in rodent cell lines, and explore the effect of selenium supplementation and deficiency on infectivity and viral fitness.

In this study, a significant ecological association between HFRS incidence and selenium deficiency was found, also after adjusting for other environmental and social-demographic variables. However, the lack of more precise data on the selenium content of crops and feed and additional data on selenium contents in soils, food grains, and the human body is a limitation of the study. A further drawback of our study is the fact that our preliminary experiments exploring a potential mechanism behind this observation did not result in a clear conclusion. Still, our results provide possible targets for focused prevention and control of HFRS (i.e. the areas of selenium deficiency with low elevation, few grasslands, or an abundance of forests). Furthermore, our findings might provide valuable information for risk evaluation of HFRS epidemics and thereby identify target populations for vaccination and health education. Additional experimental studies should further explore possible mechanisms of how selenium deficiency leads to an increased risk of HFRS. If this relationship holds, then this would provide rationale for preventive measures of HFRS, such as providing selenium supplements, either through fertilization of crops or as a preventive medicine for humans in severe selenium-deficient regions.

Materials and Methods

Epidemiological data collection and management

Since 1950, HFRS has been included in the list of notifiable infectious diseases in mainland China. Cases were reported using a standard protocol formulated by the Chinese Center for Disease Control and Prevention (CISDCP). HFRS was initially diagnosed clinically by signs and symptoms according to the guideline issued by the Ministry of Health, China. Since 1982, reported cases have also been confirmed by standard serological tests such as enzyme-linked immunosorbent assays and indirect immuno-fluorescence assays (488;518). In this study, we included all HFRS cases reported to the CISDCP during 2005 to 2010. Information on age, sex, occupation, residence address, working address, onset date and location, hospital admission date, and clinical outcomes were retrieved from the diagnostic application form sent to the reference laboratory. Demographic data of each county were obtained from the National Bureau of Statistics of China.

Information on hantaviruses infections in rodent hosts was collected from 40 surveillance sites across the country according to a protocol developed by CISDCP (519). Several investigation spots were selected within each surveillance site for surveys of rodent hosts twice every year: March-April and September-October. A total of 100-150 traps per patch with peanuts as bait were placed for 2-3 consecutive nights at each investigation spot and at least 200 rodents were caught from each surveillance site every year. Lung tissues of rodents were

collected and stored in liquid nitrogen, and then examined for hantaviruses antigens using indirect immunofluorescent assay (IFA), as previously described by Lee and others (520).

To assess the association between the number of HFRS cases and selenium deficiency, the investigation data on selenium content of crops and animal feed in various areas were obtained from the Chinese Academy of Agricultural Sciences (488;521). All counties were classified into 3 categories according to the average selenium concentration of crops including corn, barley, rice, sorghum, millet, potatoes, broad beans, wheat, etc., grown in that areas: non-selenium-deficient areas (≥ 0.06 ppm), moderate selenium-deficient areas (0.03 - 0.05 ppm), and severe selenium-deficient areas (≤ 0.02 ppm) (521). Data on geographic and social-demographic co-variables, possibly associated to the incidence of HFRS, including elevation, land use (i.e. distribution of croplands, forests and grasslands), population density, and gross domestic product (GDP), were also collected. Elevation data were derived from a shuttle radar topography mission database with a spatial resolution of 1 km (522). Data of land use and GDP were collected with a spatial resolution of 1 km from the Institute of Geographical Sciences and Natural Resources Research, Chinese Academy of Sciences. The population density of each county was obtained from the National Bureau of Statistics of China. The average elevation, proportion of area of land use, population density, and average GDP for each county were then extracted by overlapping the county boundary and each of co-variables using ArcGIS 9.2 software (ArcGIS 9.2, Environmental Systems Research Institute, Redlands, CA, USA) .

Spatial analysis of HFRS incidence

Each HFRS case was geo-referenced to the corresponding polygons of the China digital map through the linkage of the 6-digit county geo-code. The average annual incidence was calculated for each county, and a thematic map was created and overlapped onto the map of selenium concentration of crops and feed by using ArcGIS 9.2. Based on average annual incidence, all counties were grouped into four HFRS categories: non-endemic, low endemic (incidence < 5.0 per 100,000 person-years), medium endemic (5.0 to 30.0 per 100,000 person-years), and high endemic (> 30.0 per 100,000 person-years).

Ecological analysis

We used a Poisson regression to test for a relationship between incidence of HFRS and selenium deficiency. The cumulative number of HFRS cases per county was set as the outcome variable, and population size of each county was included as offset. The environmental and social-demographic factors, average elevation, proportion of areas of croplands, forests, and grasslands, population density, and average GDP for each county, were included as co-variables. For each continuous variable, we also reported the average yearly incidence of HFRS for different categories to allow inspection of the data and to assess whether the continuous assumption was justified. The categorical results for continuous variables were not included in the Poisson regression. The incidence rate ratio (IRR) in response to the change of the variable

by a given amount (1,000 m for elevation, 1000 persons per km² for population density, and 10 million Yuan per km²) was used to determine the impact of each variable on HFRS incidence. The 95% confidence interval (CI) and corresponding P-value were estimated after correcting for over dispersion, because of the nature of infectious diseases with spatial clustering patterns (522). First, univariate analyses were performed to examine the effect of each variable separately, and then multivariate analysis was performed by including all co-variables with a $P < 0.20$ in the univariate analysis. We also performed the regression analyses separately for the 5 climate regions: temperate monsoon (I), sub-tropical monsoon (II), tropical monsoon (III), highland hibernal (IV) and temperate continental (V) (523).

To verify the influence of selenium deficiency on hantavirus infection in rodents, we investigated the association between selenium content of crop and animal feed in each county and hantaviruses infection rates in the rodent hosts caught from the 40 surveillance sites in the period from 2005 to 2008. Hantaviruses infection rates were plotted against the selenium content category (non-deficient areas, moderate deficient areas and severe deficient areas). The differences in infection rates were examined using the nonparametric equality-of-medians test (524).

Clinical epidemiological study

Selenium levels in acute HFRS patients and controls

To study whether the occurrence of a HFRS case is associated with lowered circulatory selenium levels, we compared HFRS cases to non-infected healthy controls. We decided to measure the plasma activity and concentration of one of the major selenium binding proteins in human serum: glutathione peroxidase (activity of collective GPx and concentration of glutathione peroxidase -3 (GPx-3). GPx activity and GPx-3 concentration in the circulation is directly correlated to selenium concentrations (515). Blood samples of HFRS patients were collected, after informed consent, from spring 2009 to winter 2010 at the Xi'an No.8 Hospital in the Shaanxi Province, China. All cases showed conspicuous clinical signs of HFRS and whose blood samples were tested by laboratory experiment (HNTV RT-PCR positive, or IgM, or IgG HNTVG-antibody positive). Eighty-five control (non-HFRS patients and HV-antibodies negative) samples were collected among healthy volunteers in Xi'an, Shaanxi Province. All plasma samples were stored in -80 °C until use.

GPx activity was measured by commercially available Glutathione Peroxidase Assay Kit (BioVision, USA) through a coupled reaction with glutathione reductase (GR) according to the manufacturers manual. Briefly, in the assay, GPx reduce Cumene Hydroperoxide, and oxidize glutathione (GSH) to glutathione (GSSG). The generated GSSG is reduced to GSH with consumption of NADPH by GR. The decrease of NADPH is proportionally to GPx activity in the reactions. The decrease of NADPH was measured by absorbance at 340 nm, which was used to calculate the GPx Activity.

GPx3 concentration was measured using an Enzyme-linked Immunosorbent Assay Kit as by manufacturer's instructions (Uscn inc. Wuhan, China). Plasma samples were prepared in two dilutions and each dilution was measured in duplicate. Briefly, standards or plasma samples were added to the microtitre plate with pre-coated GPx3 specific antibody. The plates were washed after they were incubated at 37 °C for 2 hours. After biotinylated GPx3 antibody were added and incubated at 37 °C for 1 hour, the plates were washed again. Horseradish Peroxidase (HRP) conjugated Advin was added and incubated for 30 minutes. After the plates were washed, TMB substrate solution was added. The reaction was terminated by addition of sulfuric acid solution. The samples' absorbance was read at 450 nm using a plate reader. The GPx3 concentration was calculated by the standard curve.

***In vitro* assessment of the influence of selenium on hantavirus replication**

To study the influence of selenium concentration on hantavirus replication we studied the kinetics of the infection of an immune competent cell line (primary isolated human umbilical vein endothelial cells (HUVEC)) with a low passage hantavirus isolate. This model was chosen based on the relation between immune activation (i.e. interferon inducement) and selenium concentrations, mentioned in the introduction (506;508;515). HUVEC were harvested from fresh umbilical veins, kindly provided by the Erasmus MC birth center. Cells were isolated as described in Chapter 3.1. Briefly, Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical veins (kindly provided by the Erasmus MC birth-center). Umbilical cords were stored in sterile 500 ml PBS + gentamycin (50 µg/ml) (Leo Pharmaceutical Products, Denmark). The veins were rinsed with PBS containing 50 U/ml heparin (Leo Pharmaceutical Products). Subsequently, cells were detached with 0.1% collagenase solution (C6885, Sigma Aldrich, USA). Cell suspension was collected in a sterile 50 ml tube followed by two times centrifugation (5 minutes; 300 x g). The cell pellet was re-suspended in HUVEC medium (human endothelial-SFM medium) (Invitrogen, USA) containing 10% human serum (Lonza, the Netherlands), 20% filtrated FBS; penicilin/streptomycin 100 U/ml, 20 ng/ml fibroblast growth factor (Peprotech, USA) and 10 ng/ml of endothelial cell growth factor (Peprotech). Cell suspensions were cultured in flasks pre-coated with 20 µg/ml of fibronectin (Roche, the Netherlands). Passage two cells from one specific donor were used for this study. The identity of the endothelial cells was confirmed by flow cytometry using *Ulex europeus* lectin (EY laboratories, USA), anti-CD31 antibody (Sigma Aldrich, USA) and Von Willebrand Factor staining (Dako, the Netherlands). HUVEC were seeded into 24-well plates (Corning, U.S.A.). Confluent monolayers were infected with a multiplicity of infection (MOI) of 0.05, 1 or 3, with infectious puumala (PUUV) hantavirus freshly isolated from chronically infected bank voles (*Myodes glareolus*), kindly provided by Prof. Heikki Henttonen, METLA Forest Research Institute, University of Helsinki, Finland. Cells were incubated for 60 minutes at 37 °C in 5% CO². After incubation, the supernatant was discarded and cells were washed three times with RPMI 1640

(Gibco, Life Sciences). Subsequently, 500ul of fresh HUVEC medium was added to the wells without addition of selenium or supplemented with 200 ng/ml of cell culture suitable sodium selenium (Sigma Aldrich, the Netherlands). HUVEC were incubated for 72 hours at 37 °C in 5% CO₂. Viral replication was determined by RNA copy number quantification as described (369). The quantity of viral RNA was measured with in a real-time qRT-PCR assay (TaqMan®Fast Virus 1-Step Master Mix, Invitrogen, Life Sciences) using Applied Biosystems® 7500 Real-Time PCR system. The RNA copy number in each sample was calculated from a standard RNA curve generated by an in vitro transcribed PUUV RNA standard derived from in vitro RNA transcripts generated using a segment amplified with pan-hantavirus degenerative PCR primers as described (525).

CHAPTER 4.2

Safety and immunogenicity of an MVA-based influenza A/H5N1 vaccine.
A randomized phase I/IIa clinical trial.

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Abstract

“Background Modified Vaccinia virus Ankara (MVA) is a promising viral vector platform for H5N1 influenza vaccine development. Preclinical evaluation of MVA-based H5N1 vaccines showed their immunogenicity and safety in various animal models, warranting clinical evaluation. **Methods** In this randomized double-blind phase I/IIa study young healthy volunteers were immunized once or twice with a normal dose (10^8 plaque forming units (pfu)) or a tenfold lower dose of either the MVA-H5-sfMR (vector encoding the hemagglutinin gene of influenza A/Vietnam/1194/04 virus (H5N1 subtype) or MVA-F6-sfMR (empty vector) vaccine. Healthy volunteers that received the MVA-H5-sfMR vaccine were eligible for a boost immunization one year after the first immunization. Primary safety endpoints were local and/or systemic reactions. Secondary outcomes were hemagglutination inhibition (HI) and virus-neutralization (VN) antibody titres in the sera from the healthy volunteers. The trial is registered at the Dutch Trial Register (www.trialregister.nl) (NTR registration number: NTR3401). **Findings** 79 of the 80 healthy volunteers that were enrolled completed the study. No serious adverse events occurred. The majority of the healthy volunteers experienced one or more local and systemic reactions. Healthy volunteers that received the tenfold lower dose were prone to develop less systemic reactions. The MVA-H5-sfMR 10^8 pfu vaccine induced significantly higher antibody responses after one and two immunizations. 27 of the 39 eligible healthy volunteers were enrolled in the boost immunization study. The results indicated that a single shot MVA-H5-sfMR 10^8 pfu prime immunization resulted in higher antibody responses upon boost immunization than two shots with MVA-H5-sfMR at a ten-fold lower dose. **Interpretation** This study illustrates that the MVA-based pandemic H5N1 vaccine was well-tolerated and immunogenic and underlines that vaccine candidates arising from the MVA platform hold great promise for the future.”

Introduction

Avian influenza viruses are continuously introduced into the human population with variable impact for infected individuals and the population at large. First reports of human infection with highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype date back to 1997 (526). Since their re-occurrence in 2003, H5N1 viruses have infected at least 650 humans and 386 of these cases were fatal (527). To facilitate fast-track vaccine development for these viruses the European Medicines Agency (EMA) has developed a pandemic vaccine mock-up dossier for Europe. The A(H1N1)2009 pandemic highlighted that in spite of the possibility for fast-track licensing of conventional vaccines, the delay between onset of the pandemic and arrival of vaccines on the market proved to be unacceptable (528). Consequently the production and distribution time of vaccines in the early pandemic period still is an unsolved problem that should be taken into account in the development of candidate vaccines against future, possibly emerging H5N1 pandemic viruses (529).

The immunogenicity of H5N1 vaccine formulations based on traditional vaccine platforms is low compared to that of seasonal influenza vaccines, resulting in the need to use multiple-shot immunization regimens or the addition of an adjuvant. The limited immunogenicity is not only associated with the relative naivety of the human population towards these avian influenza viruses, but also results from an intrinsic limited immunogenicity of the H5 hemagglutinin (HA). This phenomenon was identified for various avian influenza-virus derived HA's, like H5, H7, and H9 (530). Couch et al have assessed the immunogenicity of various avian influenza vaccines and found that the secondary structures of the vaccine antigens may affect their immunogenicity (530). It is difficult to ensure consistency of the appropriate morphology when the vaccine is produced with traditional methods of virus inactivation and breakdown through treatment with detergents. Furthermore, Couch et al illustrated that in comparison with seasonal influenza virus HA's, the conformation of avian influenza HA's and their uptake and processing by immune cells is similar and does not result in limited immunogenicity. This indicates that avian influenza vaccine development could profit considerably from novel vaccine production platforms such as those based on recombinant proteins, virosomes and viral or bacterial vectors (531-534). An additional challenge for H5N1 vaccine development is the explosive antigenic diversification of avian H5N1 viruses in the past decades, which has resulted in the emergence of a plethora of virus clades, subclades and lineages (535).

Consequently a future HA-based pandemic H5N1 vaccine should be suitable for fast and large-scale production and preferably present HA in its native multimeric form. Viral vectors such as adenoviruses and poxviruses have proven to be potent antigen-presentation platforms that comply largely with these requirements (536-538). Poxviruses and more specifically Modified Vaccinia virus Ankara (MVA) are of special interest. The latter is replication-deficient in mammalian cells which is a clear advantage from a safety perspective. This will eventually allow for large-scale immunization campaigns that may also include the classical high-risk groups for

influenza. Preclinical evaluation of MVA-based H5N1 vaccines showed their immunogenicity and safety in various animal models, warranting clinical evaluation (539-544).

Here we present data from the first-in-man phase I/IIa clinical trial conducted with the MVA-HA-based H5N1 vaccine MVA-H5-sfMR assessing its safety and immunogenicity. This is the first ever demonstration that a MVA-based H5N1 candidate vaccine is safe and immunogenic in humans.

Materials & Methods

Study Design

The phase 1/2a study was randomized and double-blind performed in a single center: Erasmus Medical Center, Rotterdam, the Netherlands. The primary study objective was assessment of safety. The immunogenicity of the vaccines was assessed as a secondary objective. Our working hypothesis was that a recombinant MVA-based H5 vaccine is safe and immunogenic in humans. Both the healthy volunteers and the physicians who did the examinations and administered the vaccine were blinded for the vaccine. Eighty young adult volunteers were recruited (both male and female). When they met the inclusion and exclusion criteria (as described in the study protocol) and provided written informed consent the healthy volunteers were randomly assigned to one of the eight study arms (n=10 per arm). The arms of the study were based on the number of immunizations (one or two), the immunization dose (10^7 or 10^8 pfu) and the vaccine (MVA-H5-sfMR or MVA-F6-sfMR) (see Table 1). Sample size was calculated based on the mean antibody titres obtained in the preclinical study with a prime and boost immunization with an MVA-H5 vaccine in macaques (539).

At the first visit (week 0) blood was drawn (max 34ml) and for pregnancy was excluded within 15-30 minutes prior to the vaccine administration among women who participated. Subsequently, the healthy volunteers received one immunization, administered as a solution of 0.5mL through intramuscular injection in the deltoid muscle. Healthy volunteers were observed for one hour at the trial unit and a blood sample was taken. Then they were sent home with an ear thermometer and diary card for them to keep track of possible side-effects during seven subsequent days. In week 4 a blood sample was drawn from all healthy volunteers followed by a second immunization for the healthy volunteers in study arms 5-8. After an observation period of one hour a blood sample was taken again they were asked to complete a diary card for the next seven days. Eight and twenty weeks after their first immunization all healthy volunteers returned for their close-out visit at which the last blood sample was taken. The study design was reviewed and approved by the Central Committee on Research involving Human Subjects (CCMO) in the Netherlands. The trial is registered at the Dutch Trial Register (www.trialregister.nl) (NTR registration number: NTR3401).

Safety Assessment

The safety of the vaccine candidates was assessed using multiple tests and scores. Each subject underwent a short physical examination prior to the immunization. Heart rate, blood pressure and body temperature were registered, the injection site was examined and questions were asked on possible pre-existing conditions. This physical examination was repeated 1 hour after immunization and an additional examination was performed if necessary upon indication during the 1 hour observation period post immunization. Before and 1 hour after immunization blood was drawn to measure clinical chemical and haematological parameters in order to detect acute effects that occurred upon immunization. Four weeks after the first immunization all healthy volunteers again underwent the short physical examination and were asked by a trial physician if they had experienced any adverse reactions during and after the first week post immunization. Subsequently blood was drawn to measure the parameters as described above. Only the healthy volunteers that received a second immunization, determined by randomisation, had an additional examination and blood sampling 1 hour after immunization. To assess reactogenicity of the vaccine, the healthy volunteers received a diary card to be completed during the first seven days post immunization. In addition they received an ear thermometer to measure their body temperature twice a day (morning and evening).

Booster immunization strategy

In order to assess the possibility to boost of the H5-specific immune response, healthy volunteers that received the MVA-H5-sfMR vaccine during the main study were approached for a follow-up study (if they approved of this by informed consent). Healthy volunteers that agreed to a booster immunization received a single shot of MVA-H5-sfMR with the same dose that they received originally. Immunizations were performed 1 year after the first MVA-H5-sfMR immunization. Prior to the booster blood was drawn to determine baseline influenza virus H5-specific and MVA-IgG specific antibody responses. Healthy volunteers received a diary card to be completed during the first seven days post immunizations. Four weeks after their visit the healthy volunteers returned and blood was drawn to determine the boost-effect of the MVA-H5-sfMR immunization.

Detection of influenza virus H5-specific antibodies

Serum was obtained from blood that was collected and centrifuged in coagulation tubes (Greiner Bio-one, Alphen a/d Rijn, the Netherlands). Sera were treated with a receptor-destroying enzyme (cholera filtrate) and then heat-inactivated at 56°C. Subsequently the sera were pretreated with horse erythrocytes after which the sera were ready to be tested for the presence of anti-H5 antibodies. This was tested in the hemagglutination inhibition assay (HI) with an adapted protocol using 1% horse erythrocytes and four HA-units of either the homologous influenza H5N1 virus A/VN/1194/04 (clade 1) or the antigenically distinct strain:

A/Indonesia5/05 (A/IND/5/05) (clade 2.1). For serology, viruses were used from which the multi basic cleavage site in the HA, associated with high virulence, was deleted by reverse genetics. The use of these reverse genetics (RG) viruses in the HI assay was validated and the obtained antibody titres were comparable with those against the wild type strains (data not shown).

The sera were also tested for the presence of virus-neutralizing antibodies using a micro virus neutralization (VN) assay with the RG viruses described above. The VN assay was performed as described previously (545). Sera from MVA-H5-SFMR immunized New Zealand White Rabbits and Cynomolgus Macaques were used as a positive controls in both the HI and VN assays. For calculation purposes serum samples with an antibody titre of <10 were arbitrarily assigned a titre of 5. Seroconversion was arbitrarily defined as a post-vaccination titre of ≥ 20 or a fourfold rise in the antibody titre when the previous titre was >10. Antibody titres of ≥ 40 were arbitrarily considered to be seroprotective.

Detection of MVA-specific antibodies

Serum samples were tested for the presence of MVA-specific immunoglobulin G (IgG) antibodies. To this end, Baby Hamster Kidney-21 (BHK-21) cells (permissive for MVA virus infection) were infected with MVA-F6-sfMR at an MOI of 10 and incubated for 6-8 hours. In parallel, serum samples were preincubated with uninfected BHK-21 cells. After the incubation period serum was added to either uninfected or infected BHK-21 cells in triplicates. The cells were incubated for 16 hours at 4°C and then washed and subsequently incubated with a FITC-labelled Rabbit anti-human IgG antibody preparation (DAKO, Glostrup, Denmark). Cells were analysed by flow cytometry using a FACS Canto (BD Biosciences, Breda, The Netherlands). The fold-increase in FITC-positive cells was calculated by dividing the values from the sera incubated with the infected cells by that from the corresponding sera incubated with the uninfected cells.

Randomization and masking

Study subjects were assigned to one of the eight study groups based on a computer-generated randomization list with 10 blocks of 8 randomized study arms. The randomization code was kept by the hospital pharmacist till the end of the study period. Based on the list a generic label was generated by the hospital pharmacy that masked the content of the vaccine vial and only indicated a reference to the respective recipient. A trial nurse would check the content of the vial and would fill the syringe from the vial and provide this with the masked label to the trial physician.

Statistics

To assess statistical significance of the differences between groups the Mann-Whitney U test was performed using IBM SPSS Statistics 22. For the analysis of the HI and VN antibody titres the log₂ values of the titres were used.

Results

Study demographics

Within 2 months after recruitment start (December 2012), eighty healthy young adults, 18-28 years of age (mean= 21.9 ± 2.0), were enrolled in the study. Females comprised 61% of these volunteers. All healthy volunteers were randomly assigned to one of the eight arms of the study. The male-females ratio's per study arm are indicated in Table 1. Study immunizations began in February 2013 and the last study visits took place in July 2013. Of the eighty healthy volunteers enrolled, 79 completed the study. Thirty-nine of these healthy volunteers received one or two immunizations with the MVA-H5-sfMR vaccine and thus were eligible for inclusion in a booster immunization study that was performed one year later (January 2014). Twenty-seven healthy volunteers were enrolled in this follow-up study and received a booster immunization.

Study population		Young Healthy adults							
		Single immunization				Two immunizations			
		MVA-H5-sfMR		MVA-F6-sfMR		MVA-H5-sfMR		MVA-F6-sfMR	
Dose (pfu)		10 ⁸	10 ⁷	10 ⁸	10 ⁷	10 ⁸	10 ⁷	10 ⁸	10 ⁷
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Week 0	Visit 1	n=10 4 / 6	n=10 6 / 4	n=10 6 / 4	n=10 3 / 7	n=10 3 / 7	n=10 2 / 8	n=10 4 / 6	n=10 2 / 8
Week 4	Visit 2	n=9 ¹ 4 / 5	n=10 6 / 4	n=10 6 / 4	n=10 3 / 7	n=10 3 / 7	n=10 2 / 8	n=10 4 / 6	n=10 2 / 8
Week 8	Visit 3	n=9 4 / 5	n=10 6 / 4	n=10 6 / 4	n=10 3 / 7	n=10 3 / 7	n=10 2 / 8	n=10 4 / 6	n=10 2 / 8
Week 20	Visit 4	n=9 4 / 5	n=10 6 / 4	n=10 6 / 4	n=10 3 / 7	n=10 3 / 7	n=10 2 / 8	n=10 4 / 6	n=10 2 / 8
1 year after visit 1									
		MVA-H5-sfMR		MVA-F6-sfMR		MVA-H5-sfMR		MVA-F6-sfMR	
Dose (pfu)		10 ⁸	10 ⁷	10 ⁸	10 ⁷	10 ⁸	10 ⁷	10 ⁸	10 ⁷
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Week 0	Boost visit 1	n=5 3 / 2	n=7 4 / 3	n.a. ²	n.a.	n=6 1 / 5	n=9 2 / 7	n.a.	n.a.
Week 4	Boost visit 2	n=5 3 / 2	n=7 4 / 3	n.a.	n.a.	n=6 1 / 5	n=9 2 / 7	n.a.	n.a.

Table 1. Immunisation dosing schedules in young healthy adults

*MVA=modified vaccinia virus Ankara. MVA-H5-sfMR=vector encoding the haemagglutinin gene of influenza A/Vietnam/1194/2004 virus H5N1 subtype). MVA-F6-sfMR=empty vector. pfu=plaque-forming units. NA=not applicable because only individuals who were immunised with MVA-H5-sfMR in the first part of the study were eligible for a booster immunisation. *Individuals who received an immunisation at that timepoint. †One individual discontinued after visit 1 because of time constraints and side-effects after the first immunisation.*

Safety

No serious adverse events occurred during either the main or the follow-up study. Eleven adverse events were registered during the study period (indicated for the respective group and study period in Tables 2-4. One subject (group 6) experienced severe headache and lightheadedness during the first hour after vaccination and received paracetamol. This event may have been related to the vaccination. No further acute effects were observed during the observation period nor during the subsequent physical examination. The ten other adverse events occurred in the four weeks after immunization. One subject in group 8 reported erythema nodosum, which was unlikely but possibly related to vaccination. Five healthy volunteers (group 1, 8) reported respiratory illness and/or sore throat during the first week post immunization, which is most likely unrelated to vaccination. Their respiratory illness was accompanied by flu-like symptoms. One subject in group 5 reported a local reaction at the injection site (evening post immunization) characterized by red spots (1-2 cm in diameter). The other three healthy volunteers (group 2, 6, 7) suffered from other conditions unlikely to have a causal relation to the vaccination. Blood samples of all healthy volunteers were analyzed for the before mentioned biochemical and haematological parameters. The majority of the values were within the standard ranges (specified per sex). The deviations that were observed were either pre-existing or isolated deviations that could be attributed to the healthy volunteer's background and/or lifestyle. One subject had an elevated CK after vaccination which could have a causal relation to the vaccination, but this did not result in clinical symptoms and was not accompanied by any other biochemical deviation. Diary cards filled in for a period of seven days after each immunization recorded local and systemic reactions after the first immunization (Table 2A-B), the second immunization (Table 3A-B) and the booster immunization (Table 4A-B). The majority of the healthy volunteers reported one or more local and systemic reactions, occurring within 72 hours post immunization and the majority of the reported reactions resolved within 6 days post immunization. Focusing on the local reactions, pain at the injection site was reported most and was graded as mild-moderate by most healthy volunteers and only a minority reported the pain as severe. Itch, swelling and redness were only reported in a few cases distributed over the different groups. No difference in incidence and severity of the local reactions were experienced after the first, second or booster immunization. Most healthy volunteers experiencing systemic reactions reported them to be mild to moderate. There was

no clear difference in incidence and severity of the systemic reactions after one, two or the booster immunization. There appeared however to be a dose-response effect, as the 10⁷ pfu dose of either the MVA-H5-sfMR or MVA-F6-sfMR vaccines resulted in less reported systemic reactions than the 10⁸ pfu dose. Overall the MVA-H5-sfMR and MVA-F6-sfMR vaccines were well tolerated.

Group		1	2	3	4	5	6	7	8
Immunization		MVA-H5-sfMR 10 ⁸	MVA-H5-sfMR 10 ⁷	MVA-F6-sfMR 10 ⁸	MVA-F6-sfMR 10 ⁷	MVA-H5-sfMR 10 ⁸	MVA-H5-sfMR 10 ⁷	MVA-F6-sfMR 10 ⁸	MVA-F6-sfMR 10 ⁷
Itch	None	89% (8)	100% (10)	100% (10)	100% (10)	100% (10)	80% (8)	100% (10)	90% (9)
	mild	11% (1)	-	-	-	-	20% (2)	-	10% (1)
	moderate	-	-	-	-	-	-	-	-
	severe	-	-	-	-	-	-	-	-
Pain	None	22%(2)	40% (4)	20% (2)	70%	10%	30% (3)	50% (5)	80% (8)
	mild	22% (2)	60% (6)	40% (4)	30%	30%	50% (5)	40% (4)	20% (2)
	moderate	11% (1)	-	30% (3)	-	30%	20% (2)	10% (1)	-
	severe	45% (4)	-	10% (1)	-	30%	-	-	-
Redness	None	67% (6)	70% (7)	100% (10)	80%	90%	100% (10)	100% (10)	100% (10)
	mild	22% (2)	30% (3)	-	20%	10%	-	-	-
	moderate	11% (1)	-	-	-	-	-	-	-
	severe	-	-	-	-	-	-	-	-
	diameter¹	8.0mm	2.5mm	-	7.5mm	2.0mm	-	-	-
Swelling	None	100% (9)	90% (9)	90% (9)	90%	90%	100% (10)	90% (9)	100% (10)
	mild	-	10% (1)	10% (1)	10%	10%	-	10% (1)	-
	moderate	-	-	-	-	-	-	-	-
	severe	-	-	-	-	-	-	-	-

Table 2A: Local reactions after the 1st immunization

Group		1	2	3	4	5	6	7	8
Immunization		MVA-H5-sfMR 10 ⁸	MVA-H5-sfMR 10 ⁷	MVA-F6-sfMR 10 ⁸	MVA-F6-sfMR 10 ⁷	MVA-H5-sfMR 10 ⁸	MVA-H5-sfMR 10 ⁷	MVA-F6-sfMR 10 ⁸	MVA-F6-sfMR 10 ⁷
Rise in Bodytemp (>37.5°C)	N	56% (5)	40% (4)	70% (7)	50% (5)	70% (7)	30% (3)	40% (4)	40% (4)
	Range	37.6-38.2	37.6-38.5	37.6-39.1	37.6-38.3	37.6-39.9	37.6-39.0	37.8-39.0	37.8-39.0
	Days	1.2	3.5	2.4	2.0	1.3	2.0	1.5	1.5
Headache	None	33.3% (3)	60% (6)	30% (3)	70% (7)	10% (1)	70% (7)	60% (6)	70% (7)
	mild	33.3% (3)	20% (2)	30% (3)	10% (1)	40% (4)	-	20% (2)	10% (1)
	moderate	33.3% (3)	20% (2)	30% (3)	20% (2)	30% (3)	20% (2)	20% (2)	10% (1)
	severe	-	-	10% (1)	-	20% (2)	10% (1)	-	10% (1)
Myalgia	None	33.5% (3)	40% (4)	10% (1)	50% (5)	-	20% (2)	30% (3)	50% (5)
	mild	22% (2)	40% (4)	40% (4)	50% (5)	10% (1)	50% (5)	-	40% (4)
	moderate	11% (1)	20% (2)	30% (3)	-	60% (6)	30% (3)	60% (6)	10% (1)
	severe	33.5% (3)	-	20% (2)	-	30% (3)	-	10% (1)	-
Arthralgia	None	89% (8)	100% (10)	70% (7)	100% (10)	100% (10)	100% (10)	100% (10)	100% (10)
	mild	-	-	-	-	-	-	-	-
	moderate	-	-	20% (2)	-	-	-	-	-
	severe	11% (1)	-	10% (1)	-	-	-	-	-

Chills	None	45% (4)	90% (9)	50% (5)	100% (10)	40% (4)	80% (8)	30% (3)	70% (7)
	mild	-	-	10% (1)	-	30% (3)	20% (2)	30% (3)	20% (2)
	moderate	22% (2)	-	10% (1)	-	20% (2)	-	30% (3)	10% (1)
	severe	33% (3)	10% (1)	30% (3)	-	10% (1)	-	10% (1)	-
		(1.0 days)		(1.0 days)		(1.0 days)		(2.0 days)	
Malaise	None	22% (2)	90% (9)	20% (2)	90% (9)	40% (4)	70% (7)	50% (5)	80% (8)
	mild	33.5% (3)	-	10% (1)	-	20% (2)	10% (1)	-	10% (1)
	moderate	33.5% (3)	-	40% (4)	10% (1)	30% (3)	10% (1)	40% (4)	10% (1)
	severe	11% (1)	10% (1)	30% (3)	-	10% (1)	10% (1)	10% (1)	-
		(1.0 days)	(3.0 days)	(1.0 days)		(1.0 days)	(1.0 days)	(1.0 days)	
Fatigue	None	67% (6)	60% (6)	60% (6)	90% (9)	50% (5)	50% (5)	40% (4)	80% (8)
	mild	11% (1)	10% (1)	-	-	30% (3)	20% (2)	40% (4)	10% (1)
	moderate	-	10% (1)	30% (3)	10% (1)	20% (2)	20% (2)	20% (2)	10% (1)
	severe	22% (2)	20% (2)	10% (1)	-	-	10% (1)	-	-
		(1.0 days)	(1.5 days)	(1.0 days)			(1.0 days)		

Table 2B: Systemic reactions after the 1st immunization

<i>Group</i>		<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>
Immunization		MVA-H5- sfMR 10 ⁸	MVA-H5- sfMR 10 ⁷	MVA-F6- sfMR 10 ⁸	MVA-F6- sfMR 10 ⁷
		8/10¹	8/10	4/10²	7/10
Itch	None	90% (9)	70% (7)	90% (9)	90% (9)
	mild	10% (1)	20% (2)	10% (1)	10% (1)
	moderate	-	10% (1)	-	-
	severe	-	-	-	-
Pain	None	40% (4)	40% (4)	60% (6)	40% (4)
	mild	-	30% (3)	20% (2)	60% (6)
	moderate	30% (3)	30% (3)	20% (2)	-
	severe	30% (3)	-	-	-
		(1.0 days)			
Redness	None	70% (7)	90% (9)	70% (7)	80% (8)
	mild	30% (3)	10% (1)	30% (3)	20% (2)
	moderate	-	-	-	-
	severe	-	-	-	-
	diameter	2.0mm	2.0mm	3.0mm	2.0mm
Swelling	None	50% (5)	90% (9)	70% (7)	80% (8)
	mild	30% (3)	10% (1)	30% (3)	20% (2)
	moderate	20% (2)	-	-	-
	severe	-	-	-	-

Table 3A: Local reactions after the 2nd immunization

<i>Group</i>		<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>
Immunization		MVA-H5- sfMR 10 ⁸	MVA-H5-sfMR 10 ⁷	MVA-F6- sfMR 10 ⁸	MVA-F6- sfMR 10 ⁷ 1
Rise in Bodytemp (>37.5°C)	subjects	40% (4)	40% (4)	30% (3)	40% (4)
	Range	37.6-39.1	37.6-38.3	37.6-38.4	37.6-37.9
	Days	2.0	2.3	2.3	2.5
Headache	None	-	60% (6)	60% (6)	80% (8)
	mild	60% (6)	20% (2)	20% (2)	20% (2)
	moderate	30% (3)	10% (1)	10% (1)	-
	severe	10% (1) (1.0 days)*	10% (1) (1.0 days)	10% (1) (1.0 days)	-
Myalgia	None	30% (3)	20% (2)	20% (2)	50% (5)
	mild	40% (4)	40% (4)	40% (4)	40% (4)
	moderate	10% (1)	40% (4)	30% (3)	10% (1)
	severe	20% (2) (1.0 days)	-	10% (1) (1.0 days)	-
Arthralgia	None	90% (9)	100% (10)	90% (9)	100% (10)
	mild	-	-	10% (1)	-
	moderate	-	-	-	-
	severe	10% (1) (1.0 days)	-	-	-
Chills	None	70% (7)	100% (10)	80% (8)	100% (10)
	mild	-	-	10% (1)	-
	moderate	20% (2)	-	10% (1)	-
	severe	10% (1) (1.0 days)	-	-	-
Malaise	None	60% (6)	70% (7)	80% (8)	90% (9)
	mild	20% (2)	10% (1)	10% (1)	10% (1)
	moderate	10% (1)	10% (1)	-	-
	severe	10% (1) (1.0 days)	10% (1) (1.0 days)	10% (1) (1.0 days)	-
Fatigue	None	40% (4)	50% (5)	70% (7)	90% (9)
	mild	20% (2)	20% (2)	10% (1)	10% (1)
	moderate	30% (3)	10% (1)	20% (2)	-
	severe	10% (1) (6.0 days)	20% (2) (1.0 days)	-	-

Table 3B: Systemic reactions after the 2nd immunization. Reported sore throat and respiratory illness, started on day 1 post immunization *days in parenthesis are mean number of days reaction was reported as severe.

Group		1	2	5	6
Boost immunization		MVA-H5- sfMR 10 ⁸	MVA-H5- sfMR 10 ⁷	MVA-H5- sfMR10 ⁸	MVA-H5- sfMR 10 ⁷
		4/5	5/7	6/6¹	8/9
Itch	None	100% (5)	86% (6)	67% (4)	78% (7)
	mild	-	14% (1)	33% (2)	11% (1)
	moderate	-	-	-	11% (1)
Pain	severe	-	-	-	-
	None	40% (2)	43% (3)	-	22% (2)
	mild	40% (2)	29% (2)	17% (1)	45% (4)
Redness	moderate	20% (1)	14% (1)	33% (2)	33% (3)
	severe	-	14% (1)	50% (3)	-
	None	60% (3)	86% (6)	33% (2)	89% (8)
Swelling	mild	40% (2)	14% (1)	67% (4)	11% (9)
	moderate	-	-	-	-
	severe	-	-	-	-
diameter		10.0mm	3.0mm	2.7mm	8.0mm
Swelling	None	80% (4)	86% (6)	33% (2)	78% (7)
	mild	20% (1)	14% (1)	67% (4)	22% (2)
	moderate	-	-	-	-
severe		-	-	-	-

Table 4A: Local reactions after the boost immunization

One subject reported on his/her diary card that he/she had flu-like symptoms before the immunization *days in parenthesis are mean number of days reaction was reported as severe

Group		1	2	5	6
Boost immunization		MVA-H5- sfMR 10 ⁸	MVA-H5- sfMR 10 ⁷	MVA-H5- sfMR 10 ⁸	MVA-H5- sfMR 10 ⁷
Rise in	Subjects	100% (5)	71% (5)	50% (3)	44% (4)
Bodytemp	Range	37.7-38.5	37.6-38.2	37.6-38.2	37.6-39.0
(>37.5°C)	Days	1.6	3.2	2.7	1.3
Headache	None	40% (2)	71% (5)	-	22% (2)
	mild	40% (2)	29% (2)	16.5% (1)	33% (3)
	moderate	20% (1)	-	67% (4)	33% (3)
	severe	-	-	16.5% (4)	11% (1)
				(1.0 days)*	(1.0 days)
Myalgia	None	-	14% (1)	17% (1)	33% (3)
	mild	60% (3)	14% (1)	-	33% (3)
	moderate	40% (2)	72% (5)	33% (2)	22% (2)
	severe	-	-	50% (3)	11% (1)
				(1.0 days)	(1.0 days)
Arthralgia	None	60% (3)	86% (6)	67% (4)	78% (7)
	mild	40% (2)	-	16.5% (1)	11% (1)
	moderate	-	14% (1)	16.5% (1)	11% (1)
	severe	-	-	-	-
Chills	None	20% (1)	72% (5)	67% (4)	78% (7)
	mild	-	14% (1)	-	-
	moderate	40% (2)	14% (1)	33% (2)	22% (2)
	severe	40% (2)	-	-	-
		(1.0 days)			
Malaise	None	20% (1)	43% (3)	33% (2)	56% (5)
	mild	-	43% (3)	-	22% (2)
	moderate	60% (3)	14% (1)	50% (3)	11% (1)
	severe	20% (1)	-	17% (1)	11% (1)
		(1.0 days)		(1.0 days)	(1.0 days)
Fatigue	None	20% (1)	72% (5)	50% (3)	56% (5)
	mild	20% (1)	14% (1)	33% (2)	44% (4)
	moderate	40% (2)	14% (1)	17% (1)	-
	severe	20% (1)	-	-	-
		(1.0 days)			

Table 4B: Systemic reactions after the boost immunization

H5-specific immune responses

Pre- and post-immunization sera were tested for the presence of H5 specific antibodies in the HI and VN assay against homologous H5N1 influenza A/VN/1194/04 virus (Table 5) and heterologous H5N1 influenza A/IND/5/05 virus (Table 6). Four weeks after the first immunization antibodies were detectable against both A/H5N1 viruses and titres were higher in healthy volunteers that received the 10^8 pfu dose of MVA-H5-sfMR, groups 1 and 5 respectively. The A/VN/1194/04-specific HI GMT of the latter was significantly higher than that in groups 2 and 6, in which healthy volunteers had received the tenfold lower dose of the MVA-H5-sfMR vaccine. A second immunization (groups 5 and 6) further boosted the antibody response as measured at 8 weeks after the first immunization. HI GMT also rose for group 1, despite the lack of a second immunization, and were significantly higher than that of group 2. HI GMT of group 5 was significantly higher than that of groups 1, 2 and 6. Over time antibody titres waned as measured on time point 20 weeks, however titres for group 5 remained significantly higher than those of groups 1, 2 and 6. In the main study the maximal seroconversion rates (% of healthy volunteers with at least a four-fold increase in titre) and seroprotection rates (% of healthy volunteers with a titre of ≥ 40) were reached after two immunizations (at 8 weeks) in group 5, 100% and 80% respectively. The HI data were confirmed by those obtained with the VN assay though VN GMT generally were lower in the latter.

The extra immunization in the follow-up study resulted in a substantial boost of the antibody responses against the homologous A/VN/1194/04 (Figure 1) and heterologous A/IND/5/05 strain (Figure 2). Highest GMTs were measured for group 1 with a 100% seroconversion and 100% seroprotection rate (Appendix 2). The titres in this group were significantly higher than in group 6 in the HI assay against the A/VN/1194/04 strain and significantly higher than group 2 in the VN assay against the A/IND/5/05 strain. Also for group 2 100% seroconversion and 100% seroprotection were reached with the extra immunization.

Sera were not only tested against the homologous H5N1 influenza A/Vietnam/1194/04 virus but also against viruses from antigenically distinct clades: H5N1 influenza A/Turkey/Turkey/1/2005 virus (clade 2.2) and the transmissible variant of the H5N1 influenza A/Indonesia/5/2005 virus (clade 2.1) as described recently (451;546). Furthermore, the boosting effect was also confirmed for the antibody responses against these viruses (data not shown). Of the healthy volunteers immunized twice with MVA-F6-sfMR 10^8 pfu, three out of ten seroconverted in the HI assay (two had seroprotective antibodies titres) with a GMT of 10.1 (SD=2.8). One of ten healthy volunteers immunized twice with the 10-fold lower dose seroconverted (GMT 5.7 (SD=1.6)).

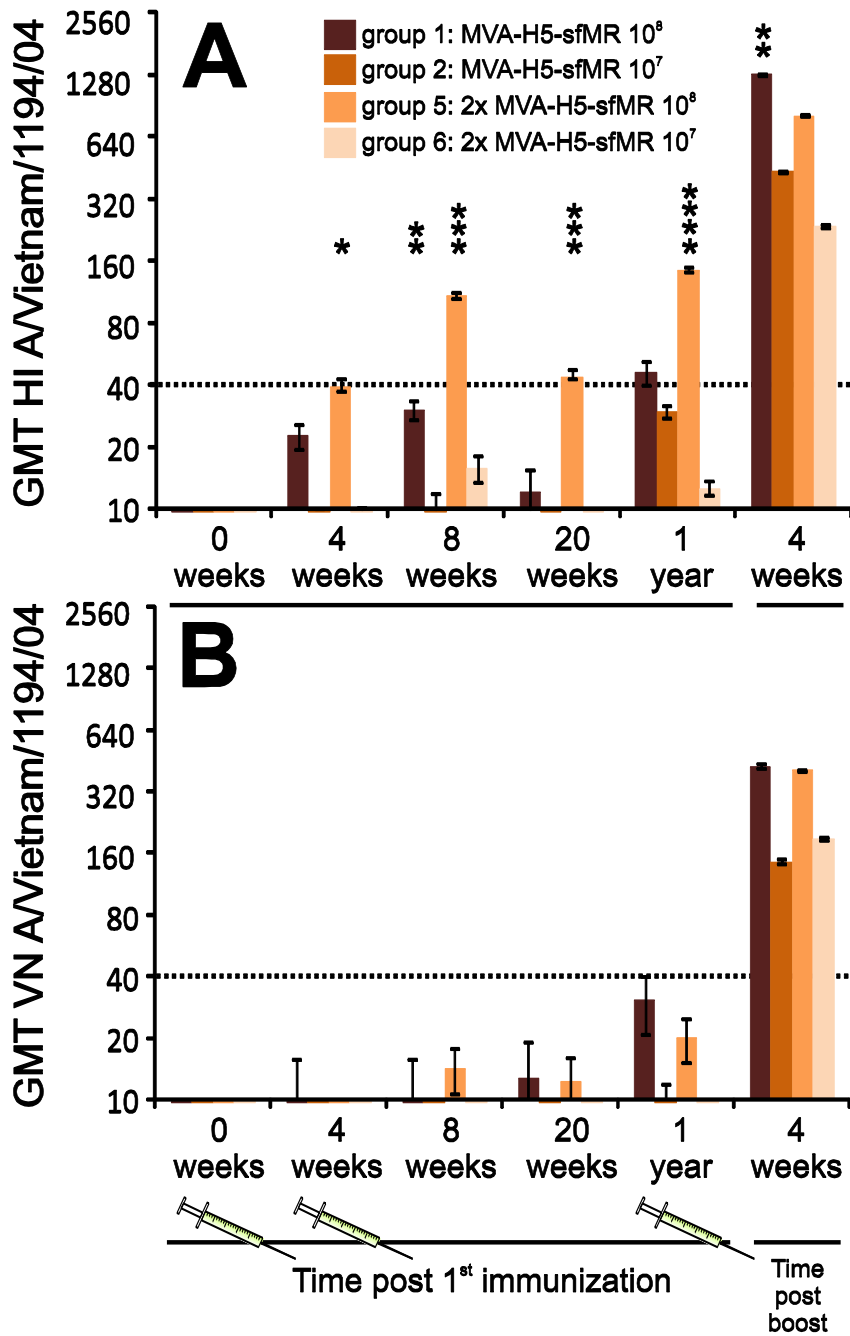


Figure 1: Mean HI (A) and VN (B) antibody titers against influenza A/Vietnam/1194/04 virus of the healthy volunteers that received a boost immunization after one year.

To assess statistical significance of the differences between groups the Mann-Whitney U test was performed. For the analysis of the HI and VN antibody titers the log₂ values were used of the titers of 0 weeks to 20 weeks for the complete groups (thus including the healthy volunteers that did not receive a booster immunization). For the 1 year and 4 weeks post boost immunization the statistics logically were performed with the boosted individuals only. *Antibody titers were significantly higher than in groups 2 and 6. ** Antibody titers were significantly higher than in group 6. *** Antibody titers were significantly higher than in groups 1, 2 and 6. **** Antibody titers were significantly higher than in group 6.

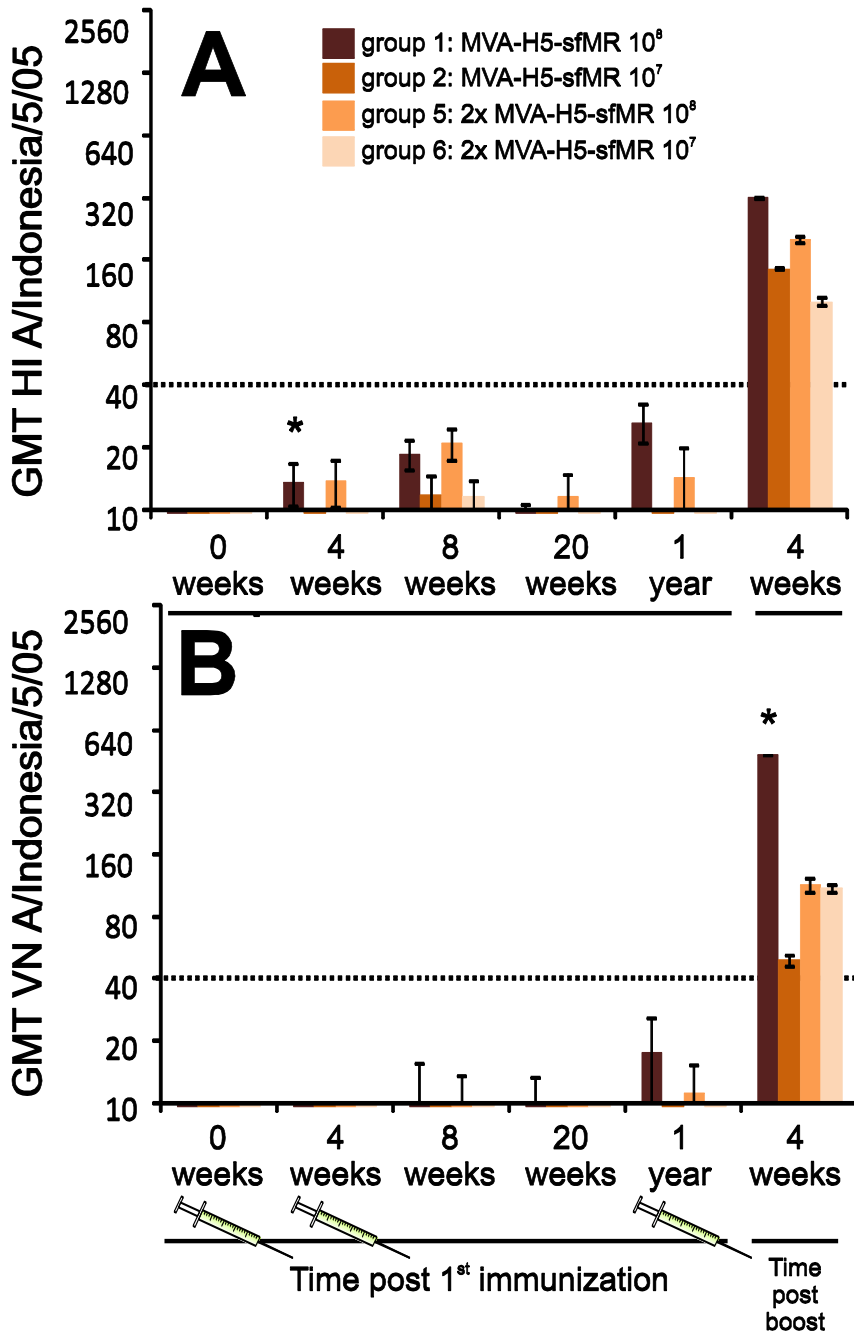


Figure 2: Mean HI (A) and VN (B) antibody titers against influenza A/Indonesia/5/05 virus of the healthy volunteers that received a boost immunization after one year. To assess statistical significance of the differences between groups the Mann-Whitney U test was performed. For the analysis of the HI and VN antibody titers the log₂ values were used of the titers of 0 weeks to 20 weeks for the complete groups (thus including the healthy volunteers that did not receive a booster immunization). For the 1 year and 4 weeks post boost immunization the statistics logically were performed with the boosted individuals only. *Antibody titers were significantly higher than in group 2 (MVA-H5-sfMR 10^7).

			<i>Time after 1st immunization</i>						
			Owks	4wks		8wks		20wks	
Group				HI	VN	HI	VN	HI	VN
1	Single shot MVA-H5-sfMR 10 ⁸ pfu	Seroconversion*	Immunization N=9	56% (5)	11% (1)	78% (7)	11% (1)	78% (7)	33% (3)
		Seroprotection		56% (5)	11% (1)	44% (4)	11% (1)	44% (4)	11% (1)
		GMT (SD)		22.6 (4.6)	9.3 (6.3)	30.2 (3.8)²	9.3 (6.3)	12.2 (3.7)	12.6 (6.3)
2	Single shot MVA-H5-sfMR 10 ⁷ pfu	Seroconversion	Immunization N=10	30% (3)	10% (1)	40% (4)	10% (1)	40% (4)	10% (1)
		Seroprotection		10% (1)	-	-	-	-	-
		GMT (SD)		8.5 (2.4)	5.7 (1.6)	9.2 (2.3)	5.0 (1.0)	6.2 (1.6)	5.0 (1.0)
5	Two shot MVA-H5-sfMR 10 ⁸ pfu	Seroconversion	Immunization N=10	80% (8)	10% (1)	100% (10)	50% (5)	100% (10)	40% (4)
		Seroprotection		60% (6)	-	80% (8)	30% (3)	70% (7)	20% (2)
		GMT (SD)		39.3 (4.1)¹	5.7 (1.6)	108.1 (2.4)³	14.1 (3.4)	43.9 (3.4)³	12.3 (3.7)
6	Two shot MVA-H5-sfMR 10 ⁷ pfu	Seroconversion	Immunization N=10	20% (2)	10% (1)	50% (5)	10% (1)	50% (5)	20% (2)
		Seroprotection		20% (2)	-	30% (3)	-	-	-
		GMT (SD)		8.1 (2.4)	5.7 (1.6)	15.8 (3.2)	5.0 (1.0)	6.9 (2.0)	5.7 (1.6)

Table 5: Cumulative seroconversion and seroprotection against homologous H5N1 virus influenza A/Vietnam/1194/2004 (clade 1)

			<i>Time after 1st immunization</i>						
			Owks	4 weeks		8 weeks		20 weeks	
Group				HI	VN	HI	VN	HI	VN
1	Single shot MVA-H5- sfMR 10 ⁸ pfu	Seroconversion*	Immunization N=9	33% (3)	11% (1)	56% (5)	11% (1)	56% (5)	22% (2)
		Seroprotection		22% (2)	-	33% (3)	11% (1)	11% (1)	11% (1)
		GMT (SD)		13.6 (3.2)¹	5.8 (1.6)	18.5 (3.1)	9.3 (6.3)	7.3 (3.2)	9.3 (4.1)
2	Single shot MVA-H5- sfMR 10 ⁷ pfu	Seroconversion	Immunization N=10	10% (1)	-	50% (5)	-	-	-
		Seroprotection		-	-	10% (1)	-	-	-
		GMT (SD)		5.7 (1.6)	-	11.8 (2.7)	-	-	-
5	Two shot MVA-H5- sfMR 10 ⁸ pfu	Seroconversion	Immunization N=10	40% (4)	10% (1)	50% (5)	20% (2)	50% (5)	20% (2)
		Seroprotection		30% (3)	10% (1)	40% (4)	20% (2)	20% (2)	10% (1)
		GMT (SD)		13.7 (3.6)	6.2 (1.9)	20.8 (3.7)	9.3 (4.1)	11.5 (3.4)	7.1 (2.1)
6	Two shot MVA-H5- sfMR 10 ⁷ pfu	Seroconversion	Immunization N=10	30% (3)	-	50% (5)	10% (1)	-	10% (1)
		Seroprotection		-	-	20% (2)	-	-	-
		GMT (SD)		7.6 (2.0)	-	11.5 (2.3)	5.7 (1.6)	-	5.0 (1.0)

Table 6: Cumulative seroconversion and seroprotection against heterologous H5N1 virus influenza A/Indonesia/5/2005 (clade 2.1)

MVA-specific IgG responses

MVA-specific IgG responses are expressed as fold increase in mean fluorescence (FIMF) (Figure 3). Four weeks after the first immunization differences between the different groups were minimal and the mean responses ranged from 1.62 to 2.1 FIMF. The second immunization for groups 5-8 induced significantly higher (p values <0.05) MVA-specific IgG responses at time point 8 weeks when compared to groups 1-4 that did not receive a second immunization (exception: the difference between group 3 and 6 was not significant). The responses waned for all groups between 8 and 20 weeks and at the latter time point MVA-specific IgG response in Groups 5, 7 and 8 were significantly higher (p values <0.05) than in Group 2. Group 7 also had a significantly higher response than Group 6 ($p=0.04$). The boost immunization for groups 1, 2, 5 and 6 resulted in a rise in MVA-specific IgG responses as measured four weeks after the booster. Differences between the groups were minimal.

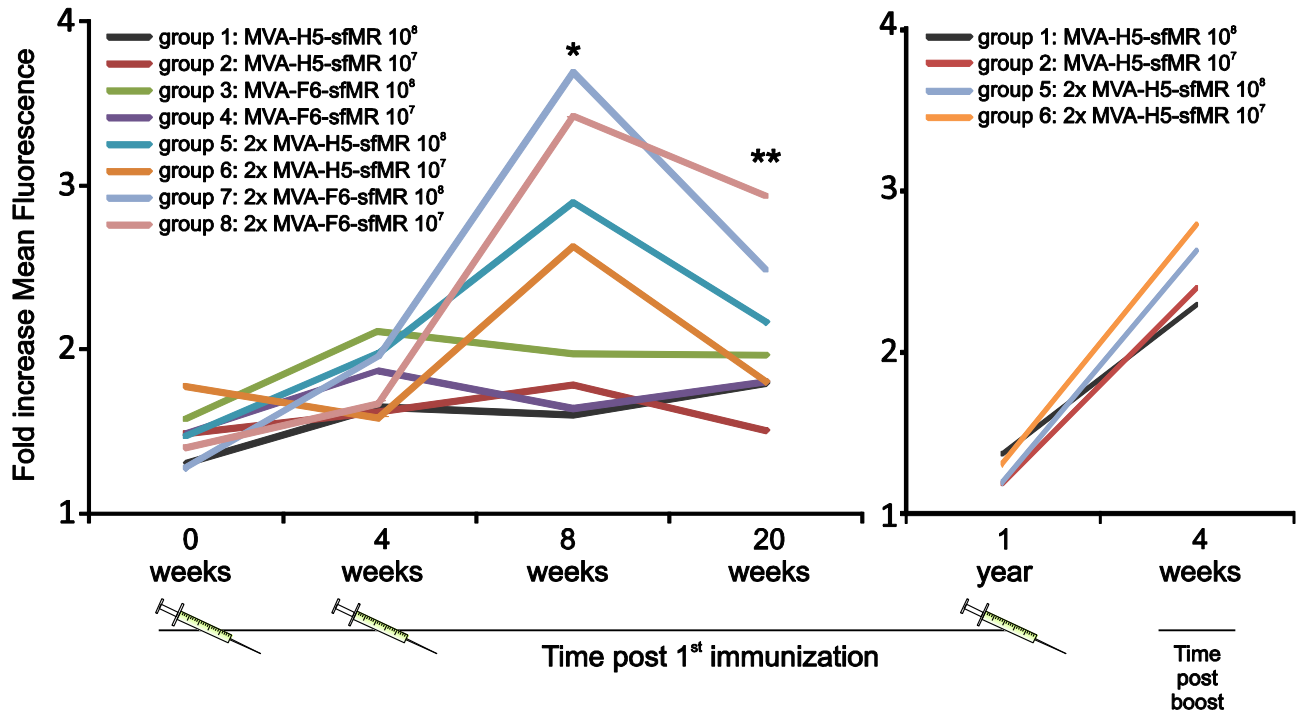


Figure 3: MVA specific antibody responses after the first, second and booster immunization.

MVA-specific IgG antibodies were measured in serum by incubating it with uninfected or MVA infected BHK-21 cells and subsequently analyzing these cells by labeling them with an anti-human IgG antibody and measuring the mean fluorescence of the cells on the FACS. MVA-specific IgG antibody responses are expressed as fold increase in mean fluorescence, calculated by dividing the values from the sera incubated with the infected cells by that from the corresponding sera incubated with the uninfected cells. To assess statistical significance of the differences between groups the Mann-Whitney U test was performed. For the analysis of the MVA-specific IgG antibody responses values were used of 0 weeks to 20 weeks for the complete

groups (thus including the healthy volunteers that did not receive a booster immunization). For the 1 year and 4 weeks post boost immunization the statistics logically were performed with the boosted individuals only. *The MVA-specific IgG response in groups 5, 6, 7 and 8 was significantly higher (p values <0.05) than in groups 1, 2, 3 and 4 (exception: the difference between group 3 and 6 was not significant). **The MVA-specific IgG response in groups 5, 7 and 8 was significant higher (p values <0.05) than in group 2. Group 7 also had a significant higher response than Group 6 ($p=0.04$).

Discussion

Here we demonstrate for the first time safety and immunogenicity of an influenza A/H5N1 candidate vaccine based on the replication deficient viral vector MVA in humans. The reactogenicity of MVA-H5-sfMR in young healthy adults was mild to moderate and apart from the limited number of adverse events, local and systemic reactions are acceptable and are in line with reports of clinical evaluations of other MVA-based vaccines (for review see (547)). The ten-fold lower dose of MVA-H5-sfMR was slightly less reactogenic than the 10^8 pfu dose, but its immunogenicity was lower. The latter induced significantly higher antibody responses, especially after two immunizations. One immunization with a dose of 10^8 pfu of MVA-H5-sfMR primed for the highest antibody responses upon boost immunization given after one year. Such a regimen would be of interest considering a pre-pandemic immunization scenario to establish priming for a H5N1-specific antibody response in a naïve population that could be boosted once a pandemic H5N1 virus would emerge.

Of interest, the antibodies induced after MVA-H5-sfMR immunization cross-reacted with influenza A/H5N1 viruses of antigenically distinct clades of H5N1 viruses, which suggests that this vaccine based on the clade 1 virus A/Vietnam/1194/04 would afford some level of protection to H5N1 viruses belonging to other clades as was demonstrated previously in mice and non-human primates (539-541).

Currently circulating H5N1 viruses do not spread efficiently from human-to-human. A handful of mutations has been identified that are associated with gain-of-function and would allow these viruses to spread from human-to-human. Most of the mutations have been identified in avian influenza viruses currently circulating in birds and it has been shown that they may accumulate upon mammalian passage of these viruses (546). This possible scenario underscores the need for the development of effective vaccines. Therefore, numerous attempts have been made to develop such vaccines. Only with high doses of conventional vaccine preparations or the use of adjuvants appreciable H5N1 virus specific antibody responses could be induced (533). Here we demonstrate that the MVA-H5-sfMR replication-deficient vector vaccine candidate was immunogenic and that especially a booster vaccination given after one year, resulted in high antibody titres. The boosting effect observed here is reminiscent of the booster effect observed with an adjuvanted heterologous H5 vaccine (548).

To compare the immunogenicity of this vaccine with conventional H5N1 inactivated (un)adjuvanted vaccine candidates head to head clinical trials are necessary.

A limitation of the use of vector-based influenza vaccines may be interference of vector-specific immunity induced by natural infections or vaccination that could affect the immunogenicity of subsequent immunizations with vaccines based on the same vector (549). The MVA-based platform does apparently not suffer from this possible drawback as was shown previously (550). In the present study we also showed that the MVA-H5-sfMR vaccine elicits strong anamnestic antibody responses to the influenza virus HA upon second and third immunizations despite the induction of anti-vector immunity upon the first immunization (549).

The boostability of the transgene encoded antigen-specific antibody response can probably be attributed to the administration of a relatively high dose of replication deficient MVA, resulting in an incomplete round of replication and co-induced expression of the transgene. The entry of MVA into the cells that eventually express and present the antigen, can apparently not be blocked by pre-existing MVA specific antibodies. This is not surprising, as antibody-mediated *in vitro* neutralization of poxviruses is notoriously inefficient and its mechanistic correlation to *in vivo* protection remains still unclear (551). T cell mediated anti-viral immunity requires at least one round of infection, gene expression and antigen presentation before becoming active and efficient recombinant antigen synthesis is possible even in the presence of strong MVA-specific CD8⁺ T cell responses (552). This aspect favours the use of this vector over replication competent viral vectors, like adenoviruses, that may suffer from pre-existing immunity against naturally occurring adenoviruses or vaccination induced vector neutralizing antibodies. The use of such vectors may have to rely on multiple serotypes and/or genetically modified variants, which could complicate practical and regulatory acceptance of the vaccine. Thus, our data show that MVA vector-specific immunity cannot abrogate the ability of the MVA-H5 vaccine to induce strong H5-specific booster responses. However, it is still unknown to what extent the priming of an H5-specific response could be modulated by anti-vaccinia immunity. This first-in-man study included only vaccinia naïve individuals and the capacity of MVA-H5 to elicit primary H5-specific immunity in healthy volunteers that were vaccinated against smallpox remains to be studied. Such follow-up studies would also inform about a potential influence of anti-vaccinia immunity on vaccine reactogenicity.

Although that this was only a single centre trial, both the safety and immunogenicity data observed in humans with the MVA-H5-sfMR candidate vaccine are in accordance with previous results that we obtained with MVA-H5 candidate vaccines in mice and non-human primates. In addition, the MVA-influenza platform could be employed for the development of vaccines against emerging influenza viruses of subtypes other than H5N1. This way a library could be established consisting of recombinant MVA vaccines, each with a prototype HA gene (e.g. H5, H7, H9, H10) that can be used as a seed virus for fast up scaling of production on a suitable

platform such as CEFs which are a well-established cell substrate that allows for large scale production of recombinant MVA viruses in a short time.

In conclusion, the present study demonstrates the favorable safety and immunogenicity profile of the MVA-based H5N1 vaccine and underlines that vaccine candidates arising from this platform hold great promise for the future.

CHAPTER 5

SUMMARIZING DISCUSSION

- 5.1** Summarizing discussion and directions for future studies
- 5.2** Nederlandse samenvatting
- 5.3** References

CHAPTER 5.1

Summarizing discussion

Summary of the main findings

Haemorrhage and thrombosis during or shortly after any infection is one of the major causes of morbidity and mortality seen in infectious diseases. The disruption of the haemostatic balance and/or disruption of the endothelial cell barrier during viral infection thereby have a major impact on human health. Further understanding of the mechanisms behind the changes in haemostatic and endothelial cell function is a necessity for the development of adequate intervention methods. Although considerable progress has been made in unravelling the mechanisms behind interactions between haemostasis, endothelial cells and certain viruses, a large part of this knowledge remains extremely limited. Furthermore lack of awareness of some of these infections related to coagulation disturbances contributes to the burden of disease of these pathogens. The studies that form this thesis can be divided in to three parts: **“Epidemiology, Pathogenesis and Prevention”**. Using the combination of epidemiological data, basal and clinical pathogenesis studies, and implementation of prevention measurements, the aim was to cover most important aspects related to (viral) infections and mechanisms of thrombosis and bleeding.

After an introduction, which provides the reader more insight into coagulation disorders in viral disease, this thesis starts with an overview of the studies related to serological evidence of circulation of neglected haemorrhagic fever pathogens in the Netherlands and Suriname. First, **Chapter 2.1** summarizes the details of a case report that led to the start of our research line focussing on hantavirus awareness and pathogenesis. The time it took to adequately diagnose the patient presented in this case and the overall lack of awareness for hantavirus infections by clinicians triggered our interest in hantavirus and subsequent rodent-borne haemorrhagic fever pathogens. The second part of **Chapter 2.1** gives more insight into these neglected pathogens of rodent-borne haemorrhagic fever and summarizes the literature. Knowledge gained by reviewing the literature facilitated the formation of the hypothesis that hantavirus disease is an underdiagnosed cause of haemorrhagic fever in the Netherlands. To do so we performed a retrospective cohort study testing sera from leptospirosis suspected, but tested negative, patients that did not travel outside of the Netherlands for the possibility of a hantavirus infection. The results of the “Hanta-Hunting” are presented in **Chapter 2.2**. Based on these results we concluded that there are a substantial number of missed cases of hantavirus infections in the Netherlands. The same strategy was applied to test a clinical cohort in Suriname. In **Chapter 2.3** we retrospectively provide strong suggestion for symptomatic

hantavirus infections in Suriname. Furthermore, we prospectively detected the introduction and emergence of the chikungunya virus to the mainland of Suriname. Phylogenetic analysis showed that the virus was most likely introduced from the Caribbean, which is still suffering from a large outbreak.

This thesis then continues with pathogenesis studies focussing on haemorrhage and clotting in infectious diseases. We attempted to determine the mechanism behind the disruption of primary and secondary haemostasis in acute Puumala hantavirus infected patients by using an *in vitro* endothelial cell model. Using this model, **Chapter 3.1** shows increased tissue factor expression and platelet binding by virus particles as possible mechanisms for developing haemorrhage. This hypothesis driven approach was the impetus for a helicopter view approach used to continue pathogenesis research. **Chapter 3.2** shows the results of proteome changes in hantavirus-infected endothelial cells. The results in **Chapter 2.1** and **Chapter 2.2** show a strong overlap in clinical signs and symptoms between haemorrhagic fever and renal syndrome (HFRS) and leptospirosis, another rodent-borne haemorrhagic fever pathogen caused by pathogenic *Leptospira*. Based on this observation pathogenic *Leptospira* were chosen as a control in this proteomic approach. Using a bacterium (*Leptospira interrogans* serovar Copenhageni) as a non-virus control with similar clinical manifestations in humans we could identify both virus specific proteins and potentially syndrome-specific candidate proteins. These data resulted in the study described in **Chapter 3.3**, and is an example of an *in vivo* – *in vitro* – *in vivo* approach. We first studied the endothelial cell response in acute leptospirosis patients and continued with laboratory experiments studying the mechanisms behind endothelial cell activation in leptospirosis. Finally, we confirmed our findings in patients again. The pathogenesis studies end with an animal (ferret) experiment to study coagulation activation in influenza virus disease in depth. Three different influenza virus strains were used, differing in virulence and clinical manifestations. **Chapter 3.4** shows activation of coagulation and fibrin deposition on the tissue level in both seasonal, pandemic and highly pathogenic avian influenza virus infection in this ferret model. The work described in the first two parts of this thesis consists of experiments with highly virulent pathogens (hantaviruses, avian influenza, chikungunya) all having a great potential for major impact on human health and welfare. Prevention remains the cornerstone of infectious disease control. In the third and final part of this thesis we focussed on disease prevention by ecology and vaccination all with the goal to minimize the burden of disease of the studied pathogens. Focussing on newly identified risk factors and prevention tools, this section of the thesis starts with **Chapter 4.1** showing the strong association between selenium deficiency and hantavirus infection incidence, both in humans and reservoir rodents. Before selenium supplementation can be implemented as a disease prevention method, many more studies are needed. This international collaborative work could be of major importance in further understanding hantavirus incidence and outbreaks, however to be so the mechanism

behind this association should be revealed. This thesis ends with an effective prevention tool in **Chapter 4.2**, listing the results of a phase I/IIa trial studying the safety and immunogenicity of a modified vaccinia Ankara virus presenting the hemagglutinin of the H5N1 avian influenza, a virus of which its pathogenicity is demonstrated in **Chapter 3.4**. Our results indicated the usefulness and effectivity of this vaccine for H5N1 while it also illustrates that this specific vector could be very suitable as a platform for vaccine production of all pathogens mentioned in this thesis.

Part I Epidemiology

Hantaviruses- widely spread, under-recognized and preventable?

This thesis starts with a summary of the clinical observations that led to the formulation of the first hypothesis tested in **Chapter 2.2**. Both the case report, discussed in the introduction of this chapter, and literature analysis of the spread, awareness and clinical manifestations of hantavirus disease, revealed certain difficulties and challenges for adequate hantavirus diagnosis. It has been described previously that hantavirus disease might be confused with dengue virus infection, leptospirosis or other viral infections, potentially leading to under-diagnosis and missed cases in other countries (553-555). To test this hypothesis for the Netherlands, we selected a cohort with symptoms and absolute certainty of no international travel history in the period before disease onset. In collaboration with the Royal Tropical Institute (KIT) we tested a cohort of leptospirosis suspected patients for the presence of hantavirus antibodies which resulted in the Hanta-Hunting study discussed in **Chapter 2.2**. Since leptospirosis diagnostics are done by manual addition of live *Leptospira* to patient sera, knowledge of the exact travel history of the patient is crucial for knowing which specific serovar is needed for the diagnostic test. This resulted in the availability of detailed information on patient history, living area and risk factors associated with environment or human behaviour. Patients that, with absolute certainty, had no chance of being potentially infected outside of the Netherlands were included in the Hanta-Hunting study. We chose a design with optimal sensitivity and decided to first screen the cohort using a pan-hantavirus Enzyme Linked Immunoabsorbant Assay (ELISA). The high seroprevalence of hantavirus antibodies in this cohort made us consider the possibility of false positive results or extensive cross reactivity in the serological diagnosis of hantavirus infections. Further confirmation by use of Immune Fluorescence Assay's (IFA) and eventually virus neutralization tests revealed a significant number of cases with absolute certainty of being a missed hantavirus case at the time of disease. The occurrence of unrecognized hantavirus cases has direct implications for clinicians. The clinical outcome of a hantavirus infection will greatly benefit from early diagnosis, which can reduce costs related to unnecessary tests and treatment. Furthermore, screening the Hanta-Hunting cohort resulted in direct implications for routine hantavirus and leptospirosis diagnostics. First of all, although a substantial number of undiagnosed hantavirus cases were discovered the Hanta-Hunting cohort also revealed a large number of false positives. This shows the need for

confirmation of unexpected results by gold standard virus neutralization tests in hantavirus epidemiological studies. The low specificity of routine hantavirus serology and subsequently the value of a single positive hantavirus test warrants further research. Also of interest is the fact that samples of leptospirosis positive patients revealed high optical density values in the commercial hantavirus ELISA tests (unpublished data). Since these results could not be confirmed by neutralization, important questions remain. First of all, papers describing possible co-infections of hantavirus and *Leptospira*, based only on ELISA or IFA test results, should be carefully analysed and in our opinion no solid conclusions should be drawn from those observations yet (553-555). Furthermore, cross reactivity between antibodies against *Leptospira* and pathogenic hantaviruses can be of interest. These two entirely different pathogens, a virus and a bacterium, colonize the same organ in rodent reservoirs and therefore co-evolution could have led to viral and bacterial proteins with great cross reactivity. These proteins can be both of importance for the usefulness of diagnostic assays but also for potential vaccine design.

The results from the Hanta-Hunting study lead to collaboration with the public health institute of Suriname (B.O.G.). We consequently tested a large fever cohort for the presence of new pathogens causing haemorrhage in Suriname. Results are discussed in **Chapter 2.3**. As in the Netherlands, a high seroprevalence of hantavirus disease was suspected on the basis of ELISA and IFA results. These results could not, however, be confirmed by virus neutralization. This illustrates the pitfall in neutralization as a gold standard test in hantavirus disease. The assay is only as strong as the (hanta)viruses available to run the test with. We made use of an “Old-World” hantavirus and tested neutralizing antibody titres against the “New-World” Andes hantavirus. The Andes virus is known to circulate in the southern parts of South America, mainly in Argentina and Chile and its reservoir is proven to be the *Oligoryzomys longicaudatus* (longtailed pygmy rice rat). Suriname is located in the northern part of South-America and rodent populations seem to differ from those reported in southern parts of South America. Since each pathogenic hantavirus is associated with one specific rodent species as reservoir host it could be that different, or even undiscovered, hantaviruses circulate in Suriname. Despite the lack of confirmation by neutralization, we do provide a strong suggestion of hantavirus circulation in Suriname. This observation warrants further attention. Molecular detection of hantaviruses in acute patients or reservoir rodents could address the presence or absence of hantavirus circulation in Suriname.

Part II Pathophysiology

Hantavirus infection, primary- and secondary haemostasis and fibrinolysis

In Part II, we changed focus to identifying disease mechanisms behind viral haemorrhagic fevers and viral infections associated with thrombosis. By using two viruses as models we aimed to further elucidate pathophysiology in the relation between viral infections and thrombosis and/or bleeding. As previously mentioned, we chose hantaviruses as a model for viral haemorrhagic fever pathogens. This is due to the fact that hantaviruses directly infect endothelial cells early in disease. This makes direct effects of the virus on endothelial function an obvious possibility in the origin of endothelial cell dysfunction (62). In Figure 2 of the introduction of this thesis we summarized the current knowledge of hantavirus infection, haemostasis and endothelial cells. Figure 1 in this discussion summarises this knowledge, now with the data and knowledge from our endothelial cell studies. The experiments executed in **Chapter 3.1** were based on a hypothesis largely formed by clinical observations in acute Puumala hantavirus patients and the strong increased risk in myocardial infarction during or shortly after Puumala infection (556). Several large cohort studies done by *Laine et al.* showed increased levels of markers related to activation of coagulation in Puumala patients. Many of these markers showed a strong correlation to disease severity (63;354). We aimed to provide insight into the mechanism behind coagulation activation by making use of a reliable and representative model. Since animal models for hantavirus disease are not accessible, hantavirus research still depends on *in vitro* experiments. Therefore, we have put much effort into the isolation of both primary endothelial cell cultures and low passage virulent Puumala hantavirus stocks. After a long design and optimization period we tested several hypothesis. Firstly, the fact that platelets are able to directly bind to PUUV particles can be of importance in further understanding primary haemostasis and its role in Puumala virus disease mechanisms. Platelets appear to be essential for the development of arterial occlusion, given that platelet aggregation inhibitors are first choice for preventive treatment in cardiovascular disease.

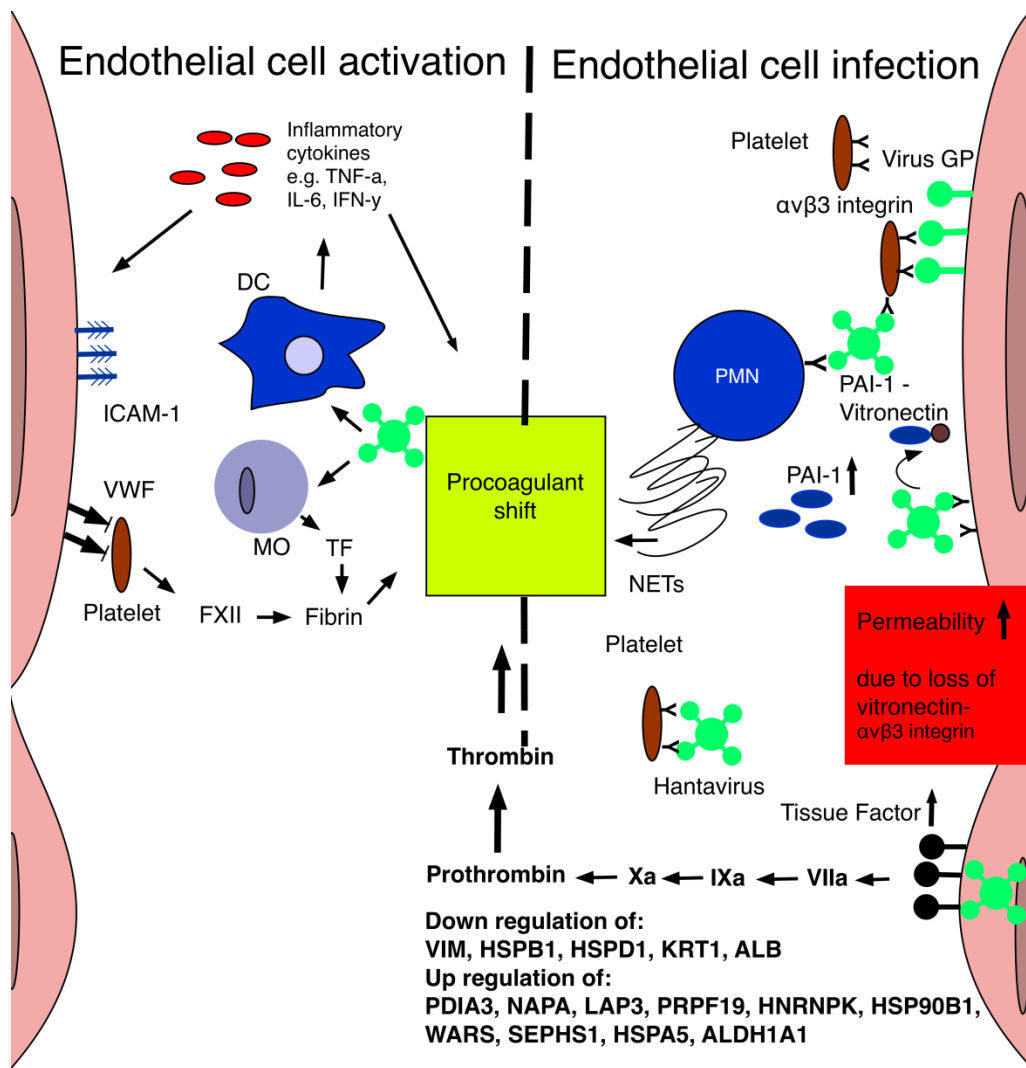


Figure 1 . Summary of, and what this thesis adds to, the knowledge of the interaction between endothelial cells and hantavirus infection

As discussed in the introduction of this thesis, the changes in haemostasis seen during hantavirus infection the result of two different pathways. At first, dendritic cells (DC) and macrophages (MO) produce pro-inflammatory cytokines during hantavirus infection promoting the procoagulant shift. Platelets bind to hantavirus infected cells via virus glycoproteins present on the infected cells. Furthermore, polymorphonuclear neutrophils (PMN) release neutrophil extracellular traps (NETs) upon binding of hantaviruses to these cells, which in turn continue to stimulate platelet aggregation and secondary coagulation. We now add to this overview that hantavirus infection of endothelial cells directly leads to an increase of tissue factor and subsequent thrombin generation. Furthermore a direct binding between Puumala hantavirus and quiescent platelets is possible. Further contributing to the procoagulant changes is the increased release of the fibrinolysis regulator plasminogen activator inhibitor type-1 (PAI-1). Possibly inhibition of fibrinolysis is further enhanced by an increase in PAI-1 stability due to the increased formation of PAI-1-vitronectin complexes during Puumala infection. Furthermore, proteome analysis of infected endothelial cells revealed multiple upregulated proteins during hantavirus infection.

We hypothesized that the binding of platelets to infected cells would coincide with an increase in VWF expression. However, antigen detection of VWF in cell lysates and quantitation of excreted VWF in supernatant of infected cells showed no alterations in VWF concentration discussed in **Chapter 3.1**. Unpublished data (Figure 2) shows quantitation of the cell surface expression of VWF on PUUV infected endothelial cells. It seems that VWF expression is actually decreased on 48 and 72 hours after infection. Since a decrease in VWF could lead to bleeding manifestations, one of the hallmark symptoms of hantavirus infections leading to HFRS, confirmation of these results could reveal interesting facts about the mechanism behind bleeding in HFRS. This is particularly so, as changes in VWF levels also influence the activity of coagulation factor eight which might have consequences for the haemostatic balance.

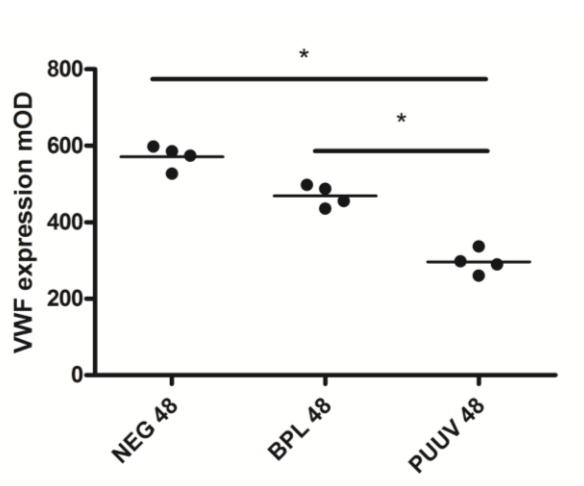


Figure 2 (unpublished data).

A decreased VWF surface expression is seen after 48 hours post infection on the surface of PUUV infected HUVEC in comparison with the negative (NEG) and inactivated (BPL) control after of Mann Whitney U test ($p = <0.05$).

Furthermore, the proteins identified in **Chapter 3.2** could give direction to future studies. Although there were fewer proteins compared to related proteomic studies of *in vitro* infection models, most of the identified proteins appeared to interact in multiple processes important for virus host interactions. Proteins such as protein disulphide isomerase, N-ethylmaleimide sensitive factor, and heterogeneous nuclear ribonucleoprotein K, have been shown to be essential in the replication and/or attachment processes of multiple viruses. Since effective anti-viral therapeutics for hantaviruses are not available, these proteins, if confirmed by siRNA or other knock down experiments, can be of interest.

Influenza

Activation of coagulation and transient consumptive coagulopathy

We finalize our work on disease mechanisms with an in-depth study of viruses associated with thrombosis. As a representative of viral infections potentially resulting in thrombosis, we evaluated the activation of coagulation in influenza virus disease. Data are presented in **Chapter 3.4**. In this chapter we describe the drastic signs of activation of coagulation in the

circulation and deposition of fibrin on the tissue level. Many of the coagulation markers correlated with markers of disease severity, but there were some discrepancies. For instance, high D-dimer levels in relative mild seasonal H3N2 infection raises questions. A systemic switch to a pro-coagulant state could theoretically add to an already present predisposition for cardiovascular disease and metaphorically speaking be the straw that breaks the camel's back (or as we would say in Dutch the drop that makes the bucket flood). Since our data are observational, further elimination experiments are needed to understand the exact mechanisms between influenza virus infection and activation of coagulation. One of the biggest questions that remain after interpreting the results of this thesis is the question why, when and how a viral infection results in either thrombosis or bleeding or possibly both. Based on our results it seems that both haemorrhagic and 'thrombotic' infections lead to pro-coagulant shift of the haemostatic balance. Whether this increased clotting tendency leads to a thrombotic or haemorrhagic event lies in very subtle differences. The correlation between coagulation markers and disease severity in influenza and the massive fibrin depositions in highly pathogenic avian influenza H5N1 virus suggest a possible relationship between the 'aggressiveness' or virulence of a virus and the coagulation response. This is in line with the observations that the severest (acute) viral infections are often associated with bleeding complications (Ebola, Marburg, dengue haemorrhagic fever etc.). It could very well be that viruses that cause a more temperate (chronic) disease (Human Immunodeficiency Virus [HIV], Cytomegalovirus [CMV]), continuously trigger the haemostatic balance towards a pro-coagulant state that, in specific cases, may lead to thrombosis. The haemorrhagic fever viruses deliver a comparable trigger to a much bigger extent, potentially resulting in consumptive coagulopathy and other pathological mechanisms associated with vascular dysfunction/bleeding. Based on our experimental work and review of the literature we strongly believe that the magnitude of the pro-coagulant trigger during viral infection, the way endothelial cells are activated (due to cytokines or direct infection) and even in which organ they are activated, all seem to play an important role in the origin of thrombosis or bleeding.

Endothelial cells and influenza virus

Another interesting comparison between hantaviruses and influenza viruses in light of haemostasis is the different role endothelial cells play in the pathogenesis of both diseases. In hantavirus infection the endothelium seems to be a target cell for direct infection resulting in endothelial dysfunction. Influenza viruses, however, have a more complicated relation with endothelial cells. Short and colleagues extensively reviewed the role of endothelial cells in influenza virus infection (557;558). They suggest that influenza virus infection of endothelial cells is most likely absent in humans and other mammals. Thus, indirect endothelial activation seems more important than direct infection of endothelial cells with influenza virus in damage to the alveolar epithelial–endothelial barrier (557). This would mean that the signs of an

activated endothelium in the ferrets tested in **Chapter 3.4** is more likely to be a secondary response to epithelial cell infection. Of interest would be to study the endothelial cell response towards infection of closely located epithelial cells, ideally under flow conditions. A final possible mechanism behind the coagulation activation in influenza virus infection is the role of neutrophils. The release of neutrophil extracellular traps (NETs) has been shown to play an important role in both ARDS and influenza virus disease. NETs are formed by chromatin fibers that neutrophils release while undergoing cell death. They have recently been shown to be an absolute necessity for the development of vascular thrombus (559). NETs can capture platelets and increase fibrin deposition (560) and thereby are very likely to contribute to pro-coagulant changes in viral infections.

Part III prevention

Prevention: “ Knowledge as Anti-Virus”

After studying the magnitude and awareness of hantavirus disease in Part I and unravelling of disease mechanisms in Part II we aimed to also put effort into prevention possibilities. Part III discusses ecology and prevention of the emerging pathogens used in the experimental studies in this thesis. The link between dietary insufficiency and hantavirus incidence was discussed in **Chapter 4.1**. The fact that low selenium levels so strongly correlate to incidence levels of HFRS in China, both in humans and rodents, warrant further in depth studies. The actual occurrence of a hantavirus infection is a multifactorial process, with many steps from reservoir rodent to human case, which can influence the occurrence (and outcome) of an infection (Figure 3). Theoretically many of these points could be influenced by decreased selenium intake. Based on our results it seems unlikely that humans with lower selenium levels are more prone to hantavirus infections. A direct mutating effect on the virus, as is described for influenza (516), due to high selenium levels could alter viral fitness and survival. However, selenium supplementation to our *in vitro* culture only mildly suppressed replication when using a very low infectious dose. The great limitation of our *in vitro* model in this case is the absence of immune cells, as many publications suggest an immunostimulant role for selenium on T-cells and macrophages in particular (507;508). Keeping the literature in mind, our experimental results discussed in **chapter 4.1**, and looking at the chain of a hantavirus infection in Figure 3, it seems most likely that selenium concentration changes have their effect in reservoir rodents. Either immune activation due to high selenium levels decreases the virus shedding in these rodents or rodents in selenium deficient areas have an increase in virus titre and subsequent shedding. This both leads to alterations in the hantavirus exposure to humans. Future experiments should determine whether the association between selenium concentration and hantavirus incidence holds true. This would potentially provide rationale for preventive

measures of HFRR, such as providing selenium supplements, either through fertilization of crops or as a preventive medicine for humans in severe selenium-deficient regions.

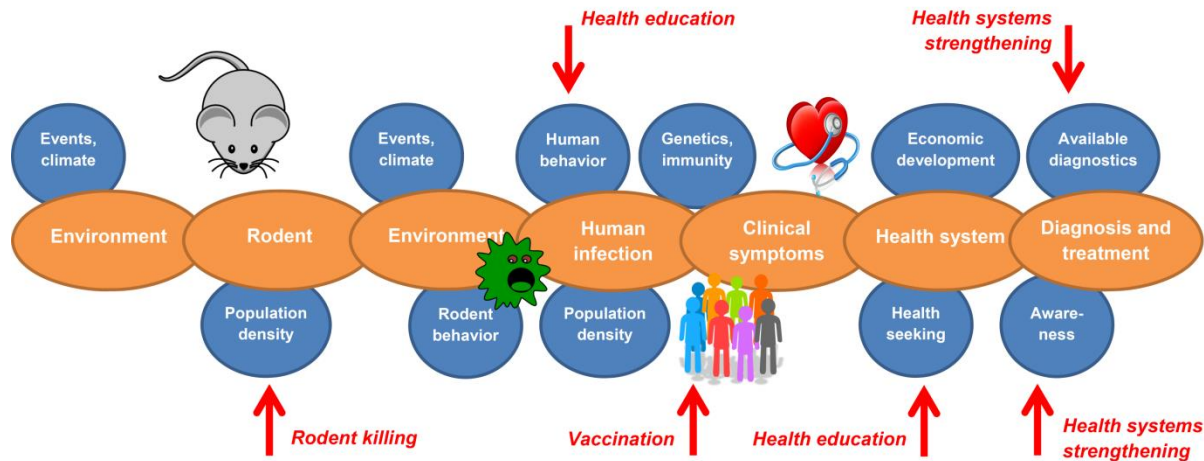


Figure 3. Chain graph of the occurrence of a hantavirus infection. Orange circles represent the steps from risk of infection in rodents to the occurrence and recognition of clinical cases. These steps can be influenced by the blue circles, while the red arrows indicate intervention possibilities. (Goeijenbier & De Vlas ©)

Another effective way of preventing infection would be by vaccinating populations at risk against hantaviruses. Currently, inactivated virus vaccines are licensed in China and Vietnam. Moreover, several new molecular vaccine approaches are in pre-clinical stages of development. However, the further development of hantavirus vaccines is largely hampered by the lack of adequate animal models and research funding (561). Another interesting possibility would be the use of viral vectors expressing hantavirus proteins from the pathogenic hantaviruses circulating in a specific area. These can be tailored to certain areas due to the stringent host restriction of pathogenic hantaviruses. In China, for instance, a vaccine protecting against Hantaan and Seoul would be of great use. In this respect the use of modified pox viruses like the Modified Vaccinia Ankara would be of great interest. Not only because of the accessible and effective technique to express viral proteins on this viral vector but also because in **Chapter 4.2** we have shown the safety and immunogenicity of such a vector.

Despite improved sanitation and the availability of effective antibiotics and vaccines, infectious diseases are still a major cause of disability, death, and socio-economic decline for millions of people. The burden of these infectious diseases is highest in lower income areas, while



knowledge and expertise related to prevention and treatment is often limited. These observations are confirmed by recent outbreaks of Ebola in West-Africa. As illustrated by Figure 3, the occurrence of a hantavirus infection is a good example of an infectious disease where prevention could benefit from counselling people at risk. Hantavirus infection can be prevented by changes in human behaviour and by reducing risk factors. To make youth aware of viral

transmission risks and adequate prevention tools we started with a project called: “Viruskenner – knowledge as anti-virus” in 2010. In the Viruskenner project, high school children are taught using tailor made teaching programs and guided by motivated young scientists. These students learn about specific characteristics of (viral) diseases that have a high impact on society. Widespread availability of knowledge and expertise can increase awareness of adequate preventative measures for young people and, in so doing, reduce the spread and impact of infectious diseases. For vector-borne disease prevention, measures like introduction and use of mosquito nets and repellent lotion, or with sex education, promoting safe sex and thereby decreasing HIV and other sexually transmitted diseases. Although these measures are available, without proper education and promotion, they will not be effective. The Viruskenner module was first tested in a small setting in the Netherlands and subsequently in Suriname. First students were taught about a specific infectious diseases. The students then created what they thought would be an effective prevention method for specific viral diseases such as HIV, dengue or hantavirus. The outcomes of a collaboration between scientists, experts of latest trends and peer communication (high school students) resulted in innovative and useful prevention tools. After the pilot phase, a large collaborative Viruskenner project has been set up this year (2015), with the project running simultaneously in the Netherlands, Suriname and Indonesia. Prospective cohort studies should reveal if this approach is a (cost)effective way to reduce the burden of infectious diseases. For now, Viruskenner is a stimulating educational method resulting in increased awareness and knowledge among groups of high school students.

Future perspectives & Concluding remarks

Coagulation disturbances play a vital role in the pathogenesis of many viral infections. Both haemorrhagic and thrombotic complications are associated with a high morbidity and mortality, confirming the essential role haemostatic imbalance plays in disease pathogenesis. For both viral haemorrhagic fever pathogens and the (often respiratory) viruses associated with vascular thrombosis further understanding of the mechanism behind this interaction and subsequent tailored treatment and/or prevention measurements will decrease the burden of these diseases. Hantaviruses proved to be a solid model for the study of mechanisms of

haemorrhage in humans. Though recently, steps have been taken toward the understanding of endothelial damage and dysfunction in hantavirus disease (53;562), the actual mechanism of how they can cause haemorrhage remains largely unknown. An interesting approach would be studying the hantavirus effects on endothelial cells of specific vascular beds. Endothelial cells have multiple important functions in vascular biology such as maintenance of haemostatic balance and the integrity of the vascular wall. Recent advances in the field of vascular biology suggest a large heterogeneity within the endothelial cells throughout the body (563). This heterogeneity of the endothelium comprises both molecular and functional features resulting in vascular bed-specific haemostasis and response to stimuli. This is shown by the fact that endothelial cells show organ- and site-specific characteristics throughout the different segments of the vascular tree adjusted to different micro-environmental conditions (564). This heterogenic functionality is further emphasized by differences in the expression of various molecules, like VE-cadherin and CD31, throughout the various vascular segments of the body (565). Furthermore, anti-coagulant responses seem to differ greatly between tissues. By regulation of the production of specific anti-coagulants a vascular bed sets its haemostatic set-point, which greatly differs between the organs. To describe this phenomenon the term vascular bed-specific haemostasis is used and seems to be the result of the interplay between extracellular signals, cell-subtype specific signalling pathways and transcriptional regulation. This could all be of importance in the mechanism behind the process that determines if a viral infection leads to thrombosis or bleeding (9). This research could be extended to studying the endothelial cell responses, assumed to be at the onset of disease in hantavirus infections, in reservoir hosts. Both homology and molecular mimicry between reservoir hosts and humans partially explains varying degrees of disease severity between different hantaviruses (53). Comparison of endothelial cell reactions in asymptomatic reservoir hosts versus the reaction observed in spill-over symptomatic hosts would give insight into the essential mechanisms of the development of hantavirus disease.

At the other end of this spectrum are the thrombotic events related to viral infection. The repeated strong association between respiratory infections and myocardial infarction warrants much more attention. Rather than confirming the association by large epidemiological studies, future studies should focus on identifying those at risk for the development of a thrombotic event and subsequent methods of prevention. It is not yet clear why some viruses have a strong influence on coagulation and are associated with thrombotic complications or bleeding, while in other viral infections this effect is limited. The complex interplay between the host, the virus (virulence), the vector and the environment (infection pressure in the community) will define clinical presentation and outcome. This might explain the different clinical presentation of viral infections in various parts of the world. Furthermore, differences in clinical presentation could also be explained by diverse tropism of viruses, such as monocytes or endothelial cells. It is not yet clear whether a pathogen associated with

bleeding or thrombosis exerts its effect through direct infection of a target cell, through pathogen-specific cross reacting antibodies, or via inflammatory mediators. In addition, inherited host factors most certainly play a role in disease severity. For implementing specific therapeutic interventions, it is crucial to know which side of the haemostatic balance the patient is on in the course of the present infection. In acute infections this is extremely difficult to determine and might be the main reason that theoretically hopeful interventions (anti-TF, activated protein C), were less successful or even disappointing in clinical practice. Before new (anti) coagulants are designed or tested we should consider testing the usability of a sensitive marker to determine the coagulation imbalance in a patient.

The proteome analysis of infected cells is a potential powerful platform in the further understanding of early host response in viral infections. To prevent the generation of large quantities of data of up and down regulated proteins, future experiments should ideally be combined with *in vivo* data and elimination experiments. By using the proteomic approach, both targets for the inhibitions of viral replication could be identified as protein changes associated with the development of disease. The latter might be a potential target for tailor made therapy in the form of protein inhibition or supplementation.

Furthermore, more knowledge is required regarding dietary insufficiencies and its effect on viral infections and potentially disease severity. The strong association between selenium concentrations and HFRS incidence warrant further attention and might hold valuable clues regarding both disease transmission and reservoir immune status. Eventually this could lead to tailor made prevention. With the continuous emergence of very severe infections like Ebola, highly pathogenic avian influenza virus and New World hantaviruses, the development of prevention strategies should be prioritized. Evaluation of the MVA platform discussed in **Chapter 4** and its suitability for immunization against hantaviruses or Ebola and Marburg would be a very favourable approach if combined with studies addressing vector immunity in the population and safety in immunocompromised patients.

CHAPTER 5.2

Nederlandse samenvatting

Hemostase en virus infecties

Het ontstaan van een bloeding of trombose gedurende of kort volgend op een infectie is een van de meest prominente oorzaken van morbiditeit en sterfte ten gevolge van infectieziekten. Hoewel aanzienlijke vooruitgang is geboekt in het ontrafelen van ziekte mechanismen in bijvoorbeeld bloedvergiftiging (sepsis) en chronische virus infecties (HIV, hepatitis B,C), is de kennis over de mechanismen die verantwoordelijk zijn voor het ontstaan van bloedingen en/of trombose ten tijde van virale infecties zeer beperkt. In dit proefschrift wordt de invloed van virale infectie op de bloedstolling bestudeerd. De focus ligt op de rol van de endotheelcel in dit proces, aangezien deze cel een belangrijke rol speelt in de regulering van de bloedstolling. Daarnaast wordt de epidemiologie van bloedende koortsen bestudeerd. Het proefschrift besluit met een overzicht van mogelijke methoden van preventie van deze veelal ernstige ziekteverwekkers. De introductie, weergegeven in **hoofdstuk 1**, geeft de lezer meer inzicht in verstoringen van de balans in de bloedstolling ten tijde van virale infecties. Er wordt een uitgebreide samenvatting gegeven met daarin een overzicht van de huidige kennis op het gebied van virus infecties en mechanismen van trombose en stolling. Tevens is er in dit hoofdstuk aandacht voor de onderzoeksgebieden waar nog kansen liggen voor de verbetering van het inzicht in de verstoring van deze balans.

Hantavirus infecties

In een groot deel van dit proefschrift ligt de nadruk op onderzoek betreffende hantavirussen. Hantavirussen zijn kleine RNA virussen die via knaagdieren worden overgedragen op mensen. Het virus bevindt zich in de uitwerpselen van deze knaagdieren en kunnen via kleine stofdeeltjes ingeademd worden. Afhankelijk van het type hantavirus kan dit leiden tot twee verschillende aandoeningen. In Europa en Azië ziet men veelal problemen met nieren en bloedstolling terwijl een hantavirus infectie in Noord- en Zuid-Amerika eerder zorgt voor het falen van zowel de longen als het hart. In beide ziektebeelden lijkt hierbij het functieverlies van de endotheelcel centraal te staan.

Directe aanleiding voor het opstarten van een hantavirus onderzoekslijn was een patiënt van wie het klinische beloop is beschreven in het case report welke de inleiding vormt van **hoofdstuk 2**. Gedurende het ziekteproces viel op dat er een lange tijd nodig was voor het stellen van de diagnose van een acute hantavirus infectie. Tevens bleek dat, in het algemeen, klinici een hantavirus infectie binnen Nederland veelal niet (direct) overwogen in hun

differentiaal diagnose. Literatuuronderzoek deed de hypothese ontstaan dat de algehele toename van hantavirus infecties in Europa, binnen Nederland niet werd opgemerkt, door het feit dat er geen gericht diagnostisch onderzoek wordt ingezet. Vervolgens is deze hypothese bevestigd in **hoofdstuk 2.2** doormiddel van het nauwkeurig nagaan van een grote patiëntengroep, verdacht van een klinisch vergelijkbare aandoening. Patiënten die op basis van symptomen of risicofactoren getest werden op de, tevens via knaagdieren overgedragen, aandoening leptospirose zijn voor deze studie uitgebreid getest op de mogelijkheid van een acute hantavirus infectie. Dit werd gedaan in de zogenaamde Hanta-Hunting studie. Doormiddel van eenzelfde strategie wordt vervolgens in **hoofdstuk 2.3** aangetoond dat er in Suriname aanwijzingen zijn voor het circuleren van hantavirussen welke bij mensen ziekte kunnen veroorzaken. Tevens faciliteerde de studie in Suriname de implementatie van diagnostische technieken waarmee de introductie en uitbraak van chikungunya in Suriname vervolgens is waargenomen. De genetische analyse liet zien dat dit virus meest waarschijnlijk geïntroduceerd is vanuit het Caribisch gebied, een gebied dat momenteel nog steeds gebukt gaat onder een grote chikungunya uitbraak.

Hantavirus interactie met endotheel

In het vervolg van het proefschrift wordt gebruik gemaakt van hantavirus infectie als model voor een bloedende koorts. Hantavirussen die voor ziekte zorgen bij mensen oefenen een vrijwel direct effect uit op endotheel cellen, al vroeg nadat zij het lichaam zijn binnen gedrongen. Onderzoek tot nu toe heeft zich vooral gericht op de invloed die het virus heeft op de doorlaatbaarheid van de endotheel barrière. In **hoofdstuk 3.1** is gebruik gemaakt van een endotheelcel model om enkele klinische observaties, gemaakt in met het Puumala hantavirus geïnfecteerde patiënten, nader te verklaren. In zieke patiënten geïnfecteerd met het Puumala hantavirus, het type virus dat in Nederland maar o.a. ook in Finland en Zweden voor ziektegevallen zorgt, laat zien dat de stollingscascade reeds in de vroege fase van de infectie geactiveerd is. Vervolgens is de mate van activatie direct gecorreleerd met de ernst van de ziekte. Infectie van vers geïsoleerde en gekweekte endotheelcellen met het Puumala hantavirus liet zien dat Tissue Factor expressie direct verhoogd werd, wat er voor zorgde dat de thrombine generatie sterk toeneemt. Daarnaast blijkt dat infectie van deze endotheelcellen er tevens toe leidt dat bloedplaatjes vastplakken aan geïnfecteerde endotheelcellen, iets wat geremd kon worden door de virusdeeltjes eerst te blokkeren met antistoffen. Uit de experimenten beschreven in dit hoofdstuk blijkt dat het Puumala hantavirus zowel de primaire als secundaire stolling direct beïnvloedt. Vervolgens is in **hoofdstuk 3.2** gekeken naar elke significante waarneembare verandering in de volledige mix van eiwitten binnen een hantavirus geïnfecteerde endotheelcel. Dit 'proteomics' experiment resulteerde in de identificatie van verschillende eiwitten die in hogere concentratie of juist lagere concentratie binnen de cel aanwezig zijn ten tijde van een hantavirus infectie vergeleken met 'gezonde' cellen. Tevens

maakten wij voor dit experiment gebruik van de bacterie *Leptospira interrogans* als extra controleconditie. Dit omdat de ziekte die veroorzaakt wordt door deze bacterie, zoals eerder vermeld, voor een soortgelijk klinisch beeld zorgt na infectie. Dit terwijl de ziekteverwekkers, een virus en een bacterie, juist zoveel van elkaar verschillen. Op deze manier hoopten wij zowel virus specifieke eiwitten te identificeren als eiwitten die specifiek zijn in het verloop van het ziekteproces. Een overeenkomst tussen beide infecties was de afname van eiwitten die essentieel zijn voor het geraamte (cytoskelet) van de endotheelcel. In **hoofdstuk 3.3** werd de interactie tussen *Leptospira* en endotheelcellen verder onderzocht waarbij een van de belangrijkste bevindingen was dat de mate van de afweerrespons van het lichaam sterk geassocieerd is met de ernst van de ziekte. Dit wekt de sterke suggestie dat niet alleen de bacterie maar tevens de respons van het lichaam hierop uiteindelijk bijdraagt aan de ernst van ziekte.

Influenza virus infecties en hemostase

Vervolgens werd de focus in dit proefschrift verlegd naar virale infecties die geassocieerd zijn met verstopping van een bloedvat; trombose. Hiervoor is gekozen voor influenza virus als model. In een diermodel hebben wij gekeken naar de mate van stollingsactivatie ten tijde van infectie met influenza virussen van verschillende mate van ziekmakend vermogen; virulentie. Zowel bij infectie met een veel voorkomende, relatief milde, seizoensgriep als tijdens infectie met de hoog pathogene H5N1 vogelgriep was de stollingscascade geactiveerd. Echter veel markers van ernst van ziekte en virologische parameters correleerden met de gemeten stollingsproducten. Dit wordt beschreven in **hoofdstuk 3.4**. De belangrijkste vraag die overblijft na afronding van dit proefschrift is hoe, in welke hoedanigheid en waar wordt bepaald of een bepaalde virale infectie resulteert in trombose, bloeding of zelfs beide. Zowel de bloedende koortsen als virus infecties, geassocieerd met trombose bestudeerd in dit proefschrift, zorgen voor een activatie van de stolling. Hoe deze stollingsactivatie uiteindelijk zal leiden tot bloeding of trombose wordt bepaald door zeer subtiele verschillen. Een belangrijke rol is weggelegd voor de virulentie, ook wel te omschrijven als de agressiviteit van het virus, iets wat gezien wordt bij de meest ernstige hemorrhagische koortsen zoals Ebola en Marburg. Het zou kunnen dat mildere of chronische infecties een weliswaar continue, maar mildere activatie van de stolling veroorzaken die uiteindelijk tot trombose zou kunnen leiden, in combinatie met andere triggers. Andere factoren die hierin een rol zouden kunnen spelen, zullen in de toekomst verder moeten worden uitgezocht. Hierbij kan men denken aan: in welk orgaan het vaatbed is aangedaan en of het endotheel direct door het virus of via de immuunrespons wordt geactiveerd.

Manieren van preventie

Het proefschrift eindigt met een tweetal hoofdstukken gericht op het voorkomen van de infecties behandeld in de eerdere delen. In **hoofdstuk 4.1** onderzochten wij omgevingsfactoren in relatie tot het voorkomen van infectieziekten. Wij toonden een sterke associatie aan tussen selenium concentratie in gewassen en de incidentie van hantavirus infecties in China. Op basis van vervolgstudies in patiënten en experimenten in het eerder beschreven endotheelcel model suggereren wij dat de variaties in selenium concentratie zeer waarschijnlijk effect hebben op de verspreiders van hantavirus infecties in China, ratten en bosmuizen. Of en hoe uiteindelijk selenium suppletie het aantal hantavirus infecties kan doen dalen, zal moeten worden uitgewezen in vervolgstudies. Aansluitend op dit hoofdstuk laten wij in **hoofdstuk 4.2** de effectiviteit en veiligheid zien van preventie door middel van vaccinatie met een gemodificeerd pokkenvirus (MVA). Op het oppervlak van dit ongevaarlijke virus is een belangrijk oppervlakte eiwit van het zeer gevaarlijke vogelgriepvirus tot expressie gebracht. Het gebruik van vector als vaccinatie biedt een veelbelovend perspectief voor bescherming tegen vele andere virussen, zoals bijvoorbeeld hantavirussen. Vervolgonderzoek zal moeten uitwijzen of brede inzet van vaccinatie middels MVA ook daadwerkelijk mogelijk is.

In dit proefschrift is enerzijds verspreiding en diagnostiek van virale bloedende koorts bestudeerd. Anderzijds werd de interactie tussen virale infecties en de stollingscascade uitgebreid onderzocht. Samengevat heeft het onderzoek beschreven in dit proefschrift bijgedragen aan de kennis van virale infecties en interactie met daarachterliggende mechanismen van trombose en bloeding. Een hantavirus infectie, met als mogelijk gevolg een bloedende koorts, is een diagnose die in ieder geval in Nederland en Suriname nog al eens over het hoofd wordt gezien. Illustratief hiervoor zijn de vele gepubliceerde patiënten casussen, waarvan één in dit proefschrift, en de resultaten van de Hanta-Hunting studie. Wat betreft de pathogenese studies lijken op het eerste gezicht zowel virussen die bloedingen veroorzaken als virussen die juist verstopping van een bloedvat kunnen veroorzaken een vergelijkbaar effect te hebben op de bloedstolling. Factoren die bepalend zijn of een virusinfectie leidt tot trombose of bloeding zullen gezocht moeten worden in zeer subtiele verschillen. Hierin lijkt dat zowel de hevigheid van de respons op infectie als het orgaan dat is aangedaan van groot belang kan zijn.

CHAPTER 5.3

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About the author

Marco Goeijenbier was born on the 17th of April 1986 in Voorburg, the Netherlands. In this city he attended the gymnasium of College 't Loo, from which he graduated in 2004. In 2004, directly after high school, Marco started medical school at the University of Amsterdam (UvA) finishing it in 2010. He developed interest for physiology and the mechanisms underlying disease already early during his studies. During his internships this interest was further encouraged by Prof.dr. E.C.M. van Gorp and dr. J.F.P Wagenaar which resulted in the first research projects at the Slotervaart hospital in Amsterdam in collaboration with the Royal Tropical Institute (KIT). In 2010 he had the opportunity to follow the Research Master of Science programme: "Infection and Immunity" at the Postgraduate School Molecular Medicine of which he graduated in 2012 'Cum Laude'. Early 2011 he started as a PhD student at the department of Viroscience of the Erasmus Medical Center in Rotterdam under supervision of Prof.dr. A.D.M.E Osterhaus, Prof.dr. E.C.M van Gorp and Dr. B.E.E Martina. Furthermore, he started working as a traveler's physician with the prior goal to advise the immune compromised patient with proper vaccination and travel policy under the supervision of Prof.dr. A. Verbon, Dr. J. Nouwen, Dr. K. Schurink and Prof.dr. E.C.M. van Gorp. In August 2015 he will start working at the department of internal medicine of the Harbour hospital (Havenziekenhuis) in Rotterdam with the goal to specialize in internal medicine.

PhD portfolio

Name PhD student	Marco Goeijenbier
Erasmus MC department	Department of Viroscience
Research school	Post-graduate molecular medicine
PhD period	2011-2015
Promotoren	Prof. dr. Eric C.M. van Gorp & Prof. dr. Albert. D.M.E Osterhaus
Co-promotoren	Dr. Byron. E.E. Martina & Dr. Jiri F.P. Wagenaar

Education

2004-2010

- Medical Degree at Universiteit van Amsterdam, Amsterdam, the Netherlands

2010-2012

- Master of Science Infection & Immunity (Cum Laude). Postgraduate school Molecular Medicine at the Erasmus Medical Center, Rotterdam, the Netherlands

In-depth courses

2011

- Study Design (NIHES)
- Scientific English writing (MolMed)
- Survival analysis (MolMed)
- Basiscursus Reizigersadviesing en immunisatie voor artsen (TravelSafe)
- Summercourse & Wintercourse I (part of Master Infection & Immunity)

2012

- The Basic Introduction Course on SPSS (MolMed)
- Summercourse & Wintercourse I (part of Master Infection & Immunity)

2013

- Course on proteomic data analysis using SameSpots[®]
- Adobe Photoshop course

2014

- Course using Ingenuity Pathway Analysis (IPA)

Presentations

Oral

2 nd Eurolepto meeting, Amsterdam, The Netherlands.	2015
3 rd International One Health congress, Amsterdam, The Netherlands.	2015
Dutch Medicine Days (FIGON), Ede, The Netherlands.	2014
AMRO meeting Amsterdam, The Netherlands.	2014
Emerging infectious diseases in Suriname, Paramaribo, Suriname.	2014
Dutch Annual Zoonoses Symposium, Utrecht, The Netherlands.	2013
Dutch Medicine Days (FIGON), Ede, The Netherlands.	2013
European congress of Virology, (ECV) Lyon, France.	2013
International Society of Thrombosis and Hemostasis, Amsterdam, The Netherlands.	2013
Comparative pathology meeting, Rotterdam, The Netherlands.	2013
Dutch Medicine Days (FIGON), Lunteren, The Netherlands.	2012
1 st Eurolepto meeting, Dubrovnik, Croatia.	2012

Poster

European Network meeting on Farm Animal Proteomics, Milan, Italy	2014
European Scientific Workgroup on Influenza, Riga, Lithuania	2014
European Society for Clinical Virology, Prague, Czech Republic	2014
International meeting on Emerging Infectious Diseases, Vienna, Austria	2013

Attended conferences and symposia

Oration Symposium prof. dr. Eric C.M. van Gorp	2013
Dutch Annual Virology Symposium, Amsterdam, The Netherlands	2013
Molecular Medicine days, Rotterdam, The Netherlands	2012
International Leptospirosis Symposium, Mérida, Mexico	2011

Teaching activities

Supervision of Msc. Thesis (SA, BB, AY, LO)	2011-2015
Co-supervision of Msc. Thesis (VK, DM)	2012-2013
Tutor 1 ^{ste} jaar geneeskunde studenten	2013
Viruskenner coach	2010-2015

Grants & Awards

Erasmus + Key action grant ANTI VIRUS	2015
ESWI young scientist grant	2014
COST Short Term Scientific Mission European Proteomic Network funding	2013
Infection & Immunity fund grant	2013
Rotterdam Global Health Initiative supportive grant	2013

Miscellaneous

Reviewer Vaccine

Reviewer Netherlands Journal of Medicine

Reviewer Plos One

Reviewer Vector Borne and Zoonotic Diseases

Reviewer BMC Infectious Diseases

Reviewer Kidney and Blood pressure research

Guest Associate Editor Frontiers in Microbiology

Publications

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* Authors contributed equally

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Euro Surveill. 2014 Aug 14;19(32).

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EC Van Gorp.
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^{*} Authors contributed equally
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2015 (1) *MT Bulletin of the Netherlands Society of Tropical Medicine*

M. Goeijenbier, A.D.M.E. Osterhaus

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Volledig volgens traditie sluit ook ik af met : “het-best-gelezen-hoofdstuk” van vrijwel elk proefschrift. Grappig, ik heb er het afgelopen jaar meermaals over nagedacht hoe ik mijn dankwoord zal gaan aanpakken en op welke originele wijze ik er een aanstekelijk stukje proza van zou kunnen maken. Echter, om te voorkomen dat dit niet alleen het best gelezen, maar tevens ook het langste hoofdstuk, van dit boekje gaat worden zal ik snel ter zake komen en proberen niet al teveel uit te wijden. Graag zou ik een hoop mensen bedanken die direct of indirect een belangrijke bijdrage hebben gehad aan de totstandkoming van het zojuist door u gelezen of bekeken proefschrift, mocht ik je, of u, tegen alle verwachtingen in niet noemen in de komende regels weet dan dat dit allerm minst met opzet is geweest en voordat ik verder ga allereerst mijn welgemeende excuses hiervoor!

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deinst risico te nemen bij het opzetten van nieuwe studies of samenwerkingen en eigenlijk overal wel kansen in ziet. Daarnaast heb ik minstens zoveel waardering voor jouw zorgzaamheid, de tijd en ruimte die je neemt voor je familie en de energie die je steekt in het bewaken van het 'groepsgevoel' binnen onze stiekem toch flink gegroeide werkgroep. Natuurlijk mag ik ook niet vergeten Inez te bedanken voor al jullie gastvrijheid en de vele fantastisch verzorgde ontbijtjes en diners ten tijde van onze vergaderingen 'op locatie'.

Dan mijn andere promotor, prof.dr. Ab Osterhaus, beste Ab, de afgelopen jaren heb jij mij continu scherp proberen en misschien zelfs weten te houden. Mijn dank is waanzinnig groot dat je het toch aandurfde met weer zo een "arts-op-het-lab" en dat ik, zo terugkijkend op de laatste vijf jaar, eigenlijk alles wat bedacht werd heb mogen uitvoeren. Jouw ontembare passie voor de wetenschap en dan met name de virologie en je continue drive naar ontdekkingen en succes zijn een ware inspiratiebron. Ik voel mij zeer bevoorrecht, al zij het als arts, werkzaam geweest te mogen zijn op het illustere "lab-van-Ab". Zoals je vele malen hebt aangegeven was de leercurve wel heel erg steil maar het lijkt dat hij nu dan toch deels beklommen is. Beste Ab, ik wil je graag bedanken voor je mentorschap en zeker voor de, soms toch niet al te malse, kritiek van de afgelopen jaren. Ik heb er enorm veel van geleerd en hoop nog even te blijven leren van je. Ik durf te wedden dat al je nieuwe initiatieven in Utrecht, Rotterdam en zelfs Hannover, stuk voor stuk een groot succes zullen worden.

Van Ab is het een logische stap naar mijn copromotor Dr. B.E.E. Martina. Beste Byron, ik hoop dat je goed beseft hoe groot jouw rol is geweest in mijn promotie. Ik ben gigantisch dankbaar voor onze samenwerking van de afgelopen jaren. Ik heb echt super veel van je geleerd, van de filosofie in een PhD, tot het zorgvuldig opbouwen van een paper en het kordaat opschrijven van de resultaten tot waar op de afdeling na 1800 nog een alcoholische versnapering verstopt kan liggen. Ik heb genoten van onze wijntjes of biertjes uiteraard geflankeerd met uitgebreide brainstorm sessies. Ik waardeer je altijd aanwezige eerlijkheid en oprechtheid, je kritische houding en continue drive naar het beter begrijpen van mechanismen in toch met name de 'exotische' virale infecties. Altijd weer als ik dol enthousiast met een (toch veelal net-niet) idee op de proppen kwam wist jij de potentieel interessante stukjes uit mijn woordenbrij te pikken en de experimenten de juiste kant op te sturen. Ik hoop dat we toch minimaal de brainstorm sessies kunnen behouden en het liefste dat we de komende jaren nog een paar hele mooie experimenten gaan uitvoeren.

Een andere rol was weggelegd voor mijn tweede copromotor, dr. J.F.P. Wagenaar. Beste Jiri, zonder jou was dit hele project wellicht nooit begonnen. Jij was degene die mij zoveel jaar geleden, met een speciaalbiertje bij Genk aan de Schinkel in Amsterdam Zuid, overhaalde gewoon nog eens te gaan praten met Eric en alle twijfels en zorgen bij hem op tafel te gooien. Nu zitten we allebei in Rotterdam en hebben we zoveel van de studies uitgevoerd waar we

destijds enkel van konden dromen. Het mooiste aan dit hele verhaal is dat eigenlijk nog veel meer van die studies moeten gaan komen en we nu de instrumenten samen in hand lijken te hebben om dit ook echt te gaan doen. Het blijven toch machtig mooie beestjes die leptospiren en met jouw enorme talent om van mijn tien zinnen twee zinnen te maken, die vervolgens nog meer zeggen zou het zonde zijn om dit niet door te zetten.

Dit boekje had vrijwel geen enkel experimenteel hoofdstuk gehad zonder de hulp van Jeroen, Petra, Cox en Fasa. Cox jij had het geduld, naja bijna altijd dan, om mij in de vroege begin periode te 'temmen' en de belangrijkste zaken van het lab uit te leggen. Daarnaast ben jij de mama van de endotheelcellen en aangezien dat voor mij toch een wel heel belangrijke cel is geworden moet ik jou heel erg dankbaar zijn voor je geduld en je kundigheid ten tijde van de navelstreng parade! Dan Jeroen, mijn grote vriend op het lab, altijd als ik iets niet van de grond kreeg maakte jij je agenda leeg en trokken we een paar dagen samen op. De hanta run-offs zijn inmiddels behoorlijk populair bij samenwerkende partijen en al jouw handige trucjes om een experiment te doen laten slagen zal ik nooit vergeten. Daarnaast ben je gewoon echt een top kerel en hebben we zoveel gelachen, van een per ongeluk geboekt homo ontmoetingshotel (jawel; de honeymoon suite), een klein roadtripje op Java van 21 uur met beide een toch behoorlijk gedesoriënteerd verteringsstelsel en vele, vele vrolijke drankjes! Ik heb echt van je genoten!

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Fasa, amigo, we spent some time in the lab together man! I must say I (almost) enjoyed every second of it. We can have so much fun together and you were always there if I needed help, however most of the times it did take half a day for you to answer your phone, but when you finally do all is fine! Thanks a lot amigo!

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Dr. Dave and now I must say !Dr.! Fokker-Nichols, you guys are so much fun to have in the room! I loved that you were full members of the exotic work group when team building activities came up. David het is enorm bewonderingswaardig hoe jij altijd iedereen maar weer bijstaat bij statistiek problematiek en minsten zo bewonderingswaardig hoe jij toch altijd net 5

minuten eerder het laatste nieuws blijkt te weten dan nu.nl of elke willekeurige andere nieuwssite. Je was een fantastische kamergenoot met boeken tips, bier tips en advies hoe om te gaan in het soms toch wat hectische klimaat van de academische jungle! Brookie, you know I think you're amazing, it was an absolute honor to be your paranimf and you have such a great family, something to be proud of, especially their participation in BEERFRIDAY. Dan zou ik direct ook al mijn (oud) kamergenoten willen bedanken, Zeli, Dineke, Widagdo, Kirsten, Coen en natuurlijk Carola en Charles. Direct zou ik dan ook mijn excuses willen aanbieden voor mijn toch licht rommelige bureaus (ja op een gegeven moment waren het er zelfs twee) in de kamer. Carola extra bedankt voor al je hulp bij eigenlijk alles, altijd sta je klaar om bij te springen, fantastisch.

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