Acta Universitatis Tamperensis 2400

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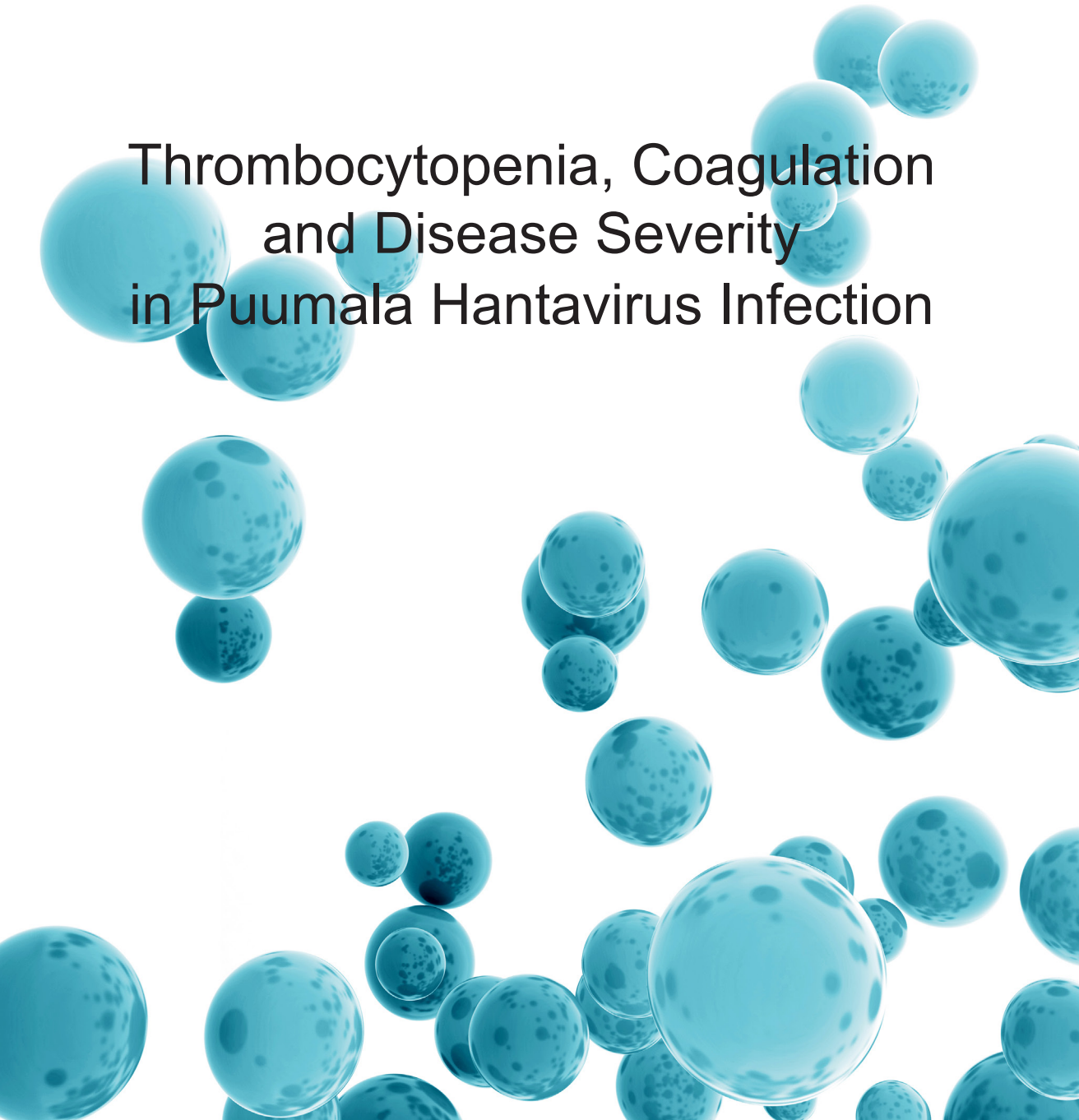
Thrombocytopenia, Coagulation and Disease Severity in Puumala Hantavirus Infection



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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty Council of the Faculty of Medicine and Life Sciences
of the University of Tampere,
for public discussion in the auditorium F114 of the Arvo building,
Arvo Ylpön katu 34, Tampere,
on 21 September 2018, at 12 o'clock.

UNIVERSITY OF TAMPERE

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Acta Universitatis Tamperensis 2400
Tampere University Press
Tampere 2018

UNIVERSITY
OF TAMPERE

ACADEMIC DISSERTATION
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Acta Universitatis Tamperensis 2400
ISBN 978-952-03-0799-8 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1909
ISBN 978-952-03-0800-1 (pdf)
ISSN 1456-954X
http://tampub.uta.fi

Suomen Yliopistopaino Oy – Juvenes Print
Tampere 2018

To my family

ABSTRACT

Puumala hantavirus (PUUV) causes a hemorrhagic fever with renal syndrome (HFRS), also named nephropathia epidemica (NE), mainly endemic in Europe and Russia. The typical clinical features of PUUV infection include thrombocytopenia, increased capillary permeability and acute kidney injury (AKI). Multiple connections between coagulation pathways and inflammation as well as complement system activation and kinin-kallikrein system have been reported. Bleeding symptoms encountered vary from mild to severe and fatal ones. Also, increased risk for cardiovascular events and venous thromboembolism have been linked to follow acute PUUV-HFRS.

Spleen size was evaluated in 20 hospitalized PUUV-infected patients by magnetic resonance imaging (MRI) in the acute and recovery phase, mean 196 days later from the onset of illness. The change of splenic length was compared with the variables describing clinical disease. Enlarged spleen was commonly detected in all patients studied, and it associated with the inflammatory laboratory markers, the C-reactive protein level and leukocyte count. Thrombocytopenia or renal impairment did not correlate with enlarged spleen.

The interactions between coagulation, endothelial activation and complement system together with the disease predictive biomarkers, pentraxin-3 (PTX-3), cell free deoxyribonucleic acid (cf-DNA), complement components SC5b-9 and C3 and interleukin-6 (IL-6) were evaluated in nineteen patients. High PTX-3 level associated with *in vivo* marker of thrombin formation, prothrombin fragments (F1+2) and with loss of physiological anticoagulants, antithrombin (AT), protein C (PC) and protein S free antigen (PS) and consumption of platelet ligand fibrinogen and reduced endothelial marker ADAMTS13.

To assess the overall hemostatic capacity of plasma during acute PUUV-HFRS, thrombin generation (TG) was measured in 27 patients by Calibrated automated

thrombography (CAT®) in the acute phase and in 23 patients in the recovery phase. Possible associations of CAT® assay with the *in vivo* measurements of thrombin formation and fibrinolysis and variables depicting disease severity were studied. The capacity of plasma to generate thrombin was decreased and hampered compared to the recovery phase. Thrombocytopenia was associated with the low endogenous thrombin potential (ETP) and low peak thrombin concentration.

The polymorphisms involved in nitric oxide (NO) production in the endothelial cells and during inflammation were investigated in 172 patients with acute PUUV infection. Patients with TT-homozygous genotype of eNOS G894T (Glu298Asp, rs1799983) polymorphism had more severe AKI, longer hospital stay, blood hemoconcentration and leukocytosis than other genotypes of this gene variant. Patients with the rare A-allele of iNOS G2087A polymorphism suffered from severe hypotension during the acute phase of infection.

TIIVISTELMÄ

Puumala hantaviruksen aiheuttamaa munuaisoireista verenvuotokuumetta, jota kutsutaan myös myyräkuumeeksi, tavataan pääasiassa Euroopassa ja Venäjällä. Myyräkuumeen tyypillisiä kliinisiä piirteitä ovat trombosytopenia, lisääntynyt kapillaarien läpäisevyys ja äkillinen munuaisvaurio. Useita hyytymisreittien ja inflammaation sekä komplementti- ja kiniini-kallikreiniini-systeemin välisiä yhteyksiä on kuvattu taudin patogeneesissa. Verenvuoto-oireet voivat vaihdella lievistä vaikeisiin ja kuolemaan johtaviin. Suurentunut kardiovaskulaarisairastavuuden ja laskimotromboosin riski on myös liitetty akuuttiin myyräkuumeen sairastamiseen.

Pernan kokoa tutkittiin magneettikuvantamisella 20 sairaalassahoidetulta, myyräkuumetta sairastavalta potilaalta taudin akuutissa vaiheessa ja toipumisvaiheessa, keskimäärin 196 päivää taudin alusta. Pernan koon muutoksen yhteyttä kliinistä taudinkuvaa kuvaaviin muuttujiin tutkittiin. Kaikilla tutkituilla potilailla perna oli lievästi suurentunut, ja se oli yhteydessä tulehduksellisiin laboratoriomuuttujiin, kohonneeseen CRP-arvoon ja negatiivisesti leukosyyttiarvoon. Trombosytopenialla tai munuaistoiminnan heikkenemisellä ei todettu korrelaatiota pernan kasvuun.

Veren hyytymisen, endoteeliaktivaation ja komplementtijärjestelmän yhteyksiä taudin ennusteellisten biomarkkereiden, kuten pentraksiini-3:n (PTX3), soluvapaan deoksiribonukleiinihapon (cf-DNA), komplementin lopputuotteen SC5b-9 ja osatekijän C3 sekä interleukiini-6:n (IL-6) välillä tutkittiin 19 myyräkuumepotilaalla. Korkea PTX3 pitoisuus oli yhteydessä trombiinin muodostumista kuvaavaan *in vivo* muuttujaan, protrombiinifragmentteihin (F1+2) ja fysiologisten antikoagulanttien, antitrombiinin, proteiini C:n aktiivisuuden ja proteiini S vapaan antigeenin vähenemiseen ja verihiutaleiden ligandin, fibrinogeenin kulutukseen sekä endoteelimuuttujan ADAMTS13-aktiivisuuden vähenemiseen.

Akuutin myyräkuumeen aikaista plasman trombiinin muodostumista tutkittiin CAT®-menetelmällä 27 sairaalassahoidetulla potilaalla akuutissa vaiheessa ja 23 potilaalla toipumisvaiheessa. Plasman kapasiteetti trombiinin tuotantoon oli vähentynyttä ja estynyttä akuutissa vaiheessa toipumisvaiheeseen verrattuna. Matala verihiutaletaso oli yhteydessä vähentyneeseen endogeeniseen trombiinipotentiaaliin (ETP) ja trombiinin huippukonsentraatioon.

Endoteelisolujen ja tulehduksen aikana typpioksidin tuotantoon osallistuvien typpioksidisyntetaasien geenipolymorfioita tutkittiin 172 potilaalla akuutissa myyräkuumeessa. Potilailla, jotka olivat TT-homotsygootteja tutkitun eNOS G894T (Glu298Asp, rs1799983) polymorfian suhteen, esiintyi vaikeampi äkillinen munuaisvaurio, pitempi sairaalaolo, enemmän veren hemokonsentraatiota ja veren valkosolutason nousua kuin muilla saman geenivariantin genotyypeillä. INOS G2087A-polymorfian harvinaista A-alleelia kantavilla myyräkuumepotilaillla esiintyi enemmän hypotensiota akuutin infektion aikana.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following four original studies, which are referred to in the text by their Roman numerals I-IV.

I Koskela S, Laine O, Paakkala A, Mäkelä S, Mustonen J: Spleen enlargement is a common finding in acute Puumala hantavirus infection and it does not associate with thrombocytopenia. Scand J Infect Dis. 2014; 46(10): 723-6.

II Laine O, Koskela S, Outinen T, Joutsi-Korhonen L, Huhtala H, Vaheri A, Hurme M, Jylhävä J, Mäkelä S, Mustonen J: Plasma pentraxin-3 and coagulation and fibrinolysis variables during acute Puumala hantavirus infection and associated thrombocytopenia. Blood Coagul Fibrinolysis 2014; 25: 612-7.

III Koskela SM, Joutsi-Korhonen L, Mäkelä SM, Huhtala H, Vaheri AI, Pörsti, Mustonen JT, Laine OK: Diminished coagulation capacity assessed by calibrated automated thrombography during acute Puumala hantavirus infection. Blood Coagul Fibrinolysis 2018; 29(1): 55-60.

IV Koskela S, Laine O, Mäkelä S, Pessi T, Tuomisto S, Huhtala H, Karhunen PJ, Pörsti I, Mustonen J: Endothelial nitric oxide synthase G894T polymorphism associates with disease severity in Puumala hantavirus infection. Plos One 2015; 10(11): e0142872.

ABBREVIATIONS

A	activated
ADAMTS13	a disintegrin and metalloproteinase with a thrombospondin type domain 13
AKI	acute kidney injury
ANDV	Andes virus
APTT	activated partial thromboplastin time
AT	antithrombin
C	complement component
CAT®	Calibrated automated thrombogram
cF-DNA	cell free deoxyribonucleic acid
CMV	cytomegalovirus
CRP	C-reactive protein
DIC	disseminated intravascular coagulopathy
DOBV	Dobrava virus
ETP	endogenous thrombin potential
eNOS	endothelial nitric oxide synthase
F1+2	prothrombin fragments
GAL-3BP	galectin-3 binding protein
GP	glycoprotein
HCPS	hantavirus cardiopulmonary syndrome
HCV	hepatitis C virus
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTNV	Hantaan virus

IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
ICAM	intercellular adhesion molecule
ISTH	the International Society of Thrombosis and Haemostasis
KDIGO	Kidney Disease: Improving Global Outcomes
LT	lag time
MAC	membrane attack complex
MRI	magnetic resonance imaging
N	nucleocapsid
NE	nephropathia epidemica
NET	neutrophil extracellular trap
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
PC	protein C
PPP	platelet poor plasma
PS	protein S free antigen
PT	prothrombin time
PTX-3	pentraxin-3
PUUV	Puumala virus
RNA	ribonucleic acid
SAAV	Saaremaa virus
SEOV	Seoul virus
SNV	Sin Nombre virus
TF	tissue factor
TGF	transforming growth factor
TG	thrombin generation
TLR	toll-like receptor
TM	thrombomodulin
TNF	tumor necrosis factor

tPA	tissue plasminogen activator
TPO	thrombopoietin
TT	thrombin time
uPA	urokinase type-plasminogen activator
VE	vascular endothelial
VEGF	vascular endothelial growth factor
VEGF-R2	vascular endothelial growth factor receptor 2
VWF	von Willebrand factor

1 INTRODUCTION

Hantaviruses can cause two acute illnesses to humans, the hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Puumala hantavirus (PUUV) causes a clinically mild form of HFRS, called nephropathia epidemica (NE) in Europe (Vapalahti et al. 2003). The virus is carried and spread by the bank vole (*Myodes glareolus*) (Brummer-Korvenkontio et al. 1980). Annually nearly 150 000 HFRS cases are estimated to be diagnosed worldwide, mainly occuring in China (Jonsson et al. 2010). Over 10 000 HFRS cases are diagnosed annually in Europe with increasing incidence. Hantaviruses have a significant impact on public health in Europe as an emerging infection (Vaheri et al. 2013a).

The clinical course of PUUV infection varies from subclinical to fatal. Typical symptoms include abrupt onset of high fever, headache, gastrointestinal symptoms, visual disturbances, hemorrhagic manifestations and acute kidney injury (AKI). Bleeding symptoms, like petechiae, epistaxis, macroscopic haematuria and melaena are present in about one third of the patients (Settergren et al. 1989). The fatality rate of PUUV infection is low, but significant acute-phase complications and long-term renal, hormonal and cardiovascular consequences can occur (Vaheri et al. 2013b, Mäkelä et al. 2010).

Thrombocytopenia is one of the main characteristics of hantavirus infection together with increased capillary permeability and acute kidney injury (Vaheri et al. 2013b). Underlying mechanisms of low platelet count suggest interactions with endothelium and platelet ligands (Laine et al. 2011). Pathogenic hantaviruses mediate the adherence of platelets on the surface of infected endothelial cells through ß3 integrins (Gavrilovskaya et al. 2010). Studies indicate active thrombopoiesis as related to an increased serum thrombopoietin (TPO) level and platelet indices (Connolly-Andersen et al. 2015, Laine et al. 2015a, Laine et al. 2016).

Endothelial activation is thought to contribute to alterations encountered in the coagulation system (Cosgriff 1991). Thrombin formation is enhanced as evaluated by decreased thrombin time and an increase in prothrombin fragments 1+2 (F 1+2), and fibrinolysis is activated (Laine et al. 2010). Approximately one fourth of the PUUV-infected patients can be diagnosed to have disseminated intravascular coagulopathy (DIC) correlating with more severe disease (Sundberg et al. 2011). The risks of cardiovascular morbidity and venous thromboembolism are suggested to be increased following acute HFRS (Connolly-Andersen et al. 2014a, Connolly-Andersen et al. 2018). Knowledge about the complex and multifactorial pathogenesis of hantaviral disease has increased in recent years (Mustonen et al. 2013). Furthermore, several biomarkers predictive of the disease severity and associated thrombocytopenia have been found (Outinen et al. 2010, Outinen et al. 2011, Sane et al. 2012, Outinen et al. 2012, Outinen et al. 2013).

This thesis is aimed to evaluate possible associations of thrombocytopenia and disease severity with the coagulation and endothelial activation. In addition, the role of the spleen during acute PUUV-HFRS was assessed. Biomarkers reflecting the disease severity of PUUV-HFRS, pentraxin-3 (PTX3), cell-free deoksiribonucleic acid (cf-DNA), interleukin-6 (IL-6) and complement proteins SC5b-9 and C3 and their associations with coagulation activation and fibrinolysis and endothelial activaton were determined. Coagulation capacity of PUUV-infected patients plasma was studied by thrombin generation assay, Calibrated automated thrombogram (CAT®). Furthermore, the role of nitric oxide (NO) synthases, with emphasis on polymorphisms of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS), and disease severity were evaluated.

2 REVIEW OF THE LITERATURE

2.1 Puumala virus and other hantaviruses

2.1.1 Virology

Hantaviruses are enveloped ribonucleic acid (RNA) viruses belonging to the *Hantavirus* genus within the *Bunyaviridae* family (Schmaljohn et al. 1985). Hantaviruses are characterized by a negative-sense three-segmented genome of approximately 12,000 nucleotides in length. The large segment encodes the viral RNA-dependent RNA-polymerase. The medium segment encodes a glycoprotein precursor, which is processed into two surface envelope glycoproteins, Gn and Gc, interacting with cell surface receptors in the viral entry and assembly (Plyusnin 2002, Cifuentes-Munoz et al 2014). Furthermore, the small segment encodes the nucleocapsid (N) protein, which encapsidates the RNA of the genome (Plyusnin 2002).

Hantaviruses replicate in the host cytoplasm and the glycoproteins are targeted to the Golgi complex, where most viruses bud. Hantaviruses infect endothelial, epithelial, macrophage, dendritic and lymphocyte cells attaching with the viral glycoprotein to the cell surface receptors. It is believed that ß1-integrin interacts with viral Gn of apathogenic hantaviruses, while ß3-integrin interacts with the glycoprotein of pathogenic hantaviruses (Gavrilovskaya et al. 1999, Mackow and Gavrilovskaya 2009). Hantaviruses can be inactivated by heat (30 min at 60°C), detergents, ultraviolet irradiation, organic solvents and hypochlorite solutions (Vapalahti et al. 2003).

Hantaviruses maintain persistent asymptomatic infection in their distinct reservoir hosts: rodents, shrews, moles and bats (Guo et al. 2013). The virus is excreted in the urine, feces and saliva of the infected animals, and due to inhalation of contaminated aerosolized excreta humans can be infected (Vapalahti et al. 2003). The reservoir host

of PUUV, the bank vole, is found in Europe with the exception of the Mediterranean region (Vapalahti et al. 2003).

Pathogenic hantaviruses can cause two diseases to humans, HFRS and HCPS (Vaheri et al. 2013a, Jonsson et al. 2010). HFRS is mainly caused by Hantaan virus (HTNV), Puumala virus (PUUV) and Dobrava virus (DOBV) in Europe and Seoul virus (SEOV) worldwide. HCPS is caused by Sin Nombre virus (SNV) and related viruses in North America, and Andes virus (ANDV) and related viruses in South America (Vaheri et al. 2013b). More than 50 hantavirus species have been discovered worldwide (Zuo et al. 2011), and 24 of those are estimated to be pathogenic strains to humans (Jiang et al. 2017).

2.1.2 Epidemiology

Over 10 000 HFRS cases are diagnosed annually in Europe with increasing numbers. PUUV, carried by the bank vole (*Myodes glareolus*), is the main cause of HFRS in Europe, following two interrelated hantaviruses, DOBV and Saaremaa virus (SAAV), carried by *Apodemus mice* (Vaheri et al. 2013a). Globally approximately 150 000-200 000 HFRS cases are estimated to occur annually, with most cases in endemic China caused by HTNV and SEOV (Jonsson et al. 2010, Bi et al. 2008). On the other hand, about 200 HCPS cases are reported every year in North and South America with an average fatality rate of 40% (Avšič-Županc et al. 2015).

The aerosol route of viral infection in humans is the most common, although person-to-person transmission of ANDV has been reported in an HCPS outbreak in Argentina (Wells et al. 1997). The seroprevalence of population in Europe is quite considerable, although the reported HFRS cases remain few. In addition, epidemiological data is lacking from many countries (Vaheri et al. 2013a). Globally hantaviruses are considered as emerging zoonotic pathogens with a significant impact on public health.

PUUV was detected in bank voles in Finland in 1980, although NE was known in Fennoscandia since the early 1930`s (Brummer-Korvenkontio et al. 1980). In a Finnish study, a total of 30 942 PUUV infection cases were reported between 1995 and 2014. The highest annual incidence was 31 cases/100 000 patient-years in Eastern Finland (Sane et al. 2016). The annual incidence has been 1000-3000 cases reported in the recent years (Vaheri et al. 2013a). The peak of PUUV infections reflect the abundance of bank vole populations and climate following three to four year cycles (Sane et al. 2016). The epidemic usually starts in the late summer, when people get infected during summer vacations. The major peak is from November to February after the major bank vole density peak, and when the rodents have entered human dwellings (Vapalahti et al. 2003, Vaheri et al. 2013b). In Finland and northern Sweden the PUUV seroprevalence is about 5% (Jonsson et al. 2010).

2.2 Hantaviral disease

2.2.1 Clinical features of PUUV infection

The clinical picture of HFRS varies from subclinical to fatal outcome. The incubation period of PUUV infection is 2-6 weeks (Vaheri et al. 2013a). The clinical course is typically divided into febrile, hypotensive, oliguric, diuretic and convalescent phases, but these phases may not be clinically evident (Vapalahti et al. 2003). PUUV infection starts abruptly with high fever and headache followed by gastrointestinal symptoms, nausea, vomiting, abdominal pain and backache (Settegren et al. 1988, Vapalahti et al. 2003). The typical clinical phases, symptoms and complications of HFRS are presented in Table 1.

Ocular symptoms are very common (70%). Decreased intraocular pressure and myopic shift mainly due to thickening of the lens are observed during acute phase of PUUV-HFRS (Hautala et al. 2011). In addition, somnolence, dizziness and other symptoms of central nervous system are common. In a Finnish study with 58 PUUV-

infected patients, 87% experienced cenral nervous system symptoms, and in half of the cerebrospinal fluid samples signs of inflammation and PUUV-IgM were detected (Hautala et al. 2010). Hypotension or shock and pulmonary oedema may develop rapidly in severe cases (Mustonen et al. 1994a).

The kidney disease in PUUV-HFRS including transient proteinuria, microscopic hematuria and AKI usually begins on the third or fourth day of the illness. In the second week oliguria is followed by polyuria and spontaneuos recovery of renal function. Up to 6% of the hospitalized PUUV patients need transient hemodialysis treatment (Lähdevirta 1971, Settegren et al. 1989, Mustonen et al. 2013). AKI, as evaluated by elevated serum creatinine level, is found in 84% hospital-treated patients (Mustonen et al. 2017).

Typical renal histological finding is acute tubulointerstitial nephritis. Hantaviruses can infect tubular epithelial cells, glomerular endothelial cells and podocytes of human kidney, and disrupt cell-to-cell contacts in these cells. This is supposed to diminish the barrier function of the kidney, and thus is the direct cause of proteinuria (Krautkrämer et al. 2011). Lack of histological endothelial cell damage suggests, that the loss of barrier function is due to infection or cytokines instead of endothelial cell death (Kanerva et al. 1998a, Vaheri et al. 2013b). A high degree of proteinuria is a special feature of PUUV associated tubulointerstitial nephritis (Mustonen et al. 2017). Several biomarkers for predicting severe AKI have been recently described (Vaheri et al. 2013a, Mustonen et al. 2017).

Hantaviral disease is considered to be rare in children, and the clinical course is similar but milder than in adults (Mustonen et al. 1994b, Huttunen et al. 2011). Full recovery is usual, but long-term consequinces may include renal impairment and hypertension (Mäkelä et al. 2000, Miettinen et al. 2006). In addition, hormonal deficiencies, such as hypopituitarism, primary hypothyroidism and testicular failure have been described after PUUV-HFRS (Mäkelä et al. 2010). The case-fatality rate varies from 0.1% up to 0.4% (Hjertqvist et al. 2010, Mustonen et al. 2013).

2.2.2 Hemorrhagic findings

Bleeding symptoms, which are seen in approximately one third of the patients, remain commonly mild. Petechiae of skin and mucosa (12%), ecchymoses, conjuctival bleedings and epistaxis (11-21%) are reported (Lähdevirta 1971, Settegren et al. 1989). In addition conjunctival bleeding, metrorrhagia, macroscopic hematuria, melena and hematemesis occur (Settegren et al. 1989). Gastrointestinal bleeding was reported by gastroscopy in all studied ten Finnish patients (Nuutinen et al. 1992). Furthermore, severe and even fatal hemorrhages of pituitary gland, kidneys, heart, liver, lungs and peritoneal cavity have been described (Valtonen et al. 1995, Hautala et al. 2002). Also a case of spleen hemorrhage has been reported (Alexeyev et al. 1994).

In DOBV infections hemorrhagic complications are more common (26-59 %), and thrombocytopenia is more severe than in PUUV infections (Vapalahti et al. 2003). For HTNV, serious hemorrhages occur in 20-30 % of the patients (Jonsson et al. 2010), and almost all patients (94%) have macroscopic bleedings (Zhang et al. 2011). SEOV causes milder bleeding manifestations compared to HTNV. In a study with 70 Brazilian HCPS cases, mild hemorrhages such as hematuria, hematemesis, intestinal bleeding and metrorrhagia was encountered in half of the patients (Jonsson et al. 2010).

Table 1. Typical clinical phases, symptoms and complications of HFRS. Modified from Jiang et al. 2016.

Phase of disease	**Febrile**	**Hypotensive**	**Oliguric**	**Polyuric**	**Covalescent**
Time of occurrence	1-7 days	1-3 days	2-6 days	2 weeks	3-6 months
Typical features	fever	hypotension	decreased urine output	increased urine output	
Symptoms	Headache Vomiting Abdominal pains Back pains Visual disturbances	Capillary leakage	Anuria (urine output < 100ml/day)	Weight decrease	Weakness Fatigue

Complications: Bleedings, DIC, acute encephalomyelitis, multiorgan failure, pituitary hemorrhage, glomerulonephritis, pulmonary edema, shock, ARDS

2.2.3 Diagnosis

HFRS can be suspected on the basis of clinical picture, although the diagnosis should be serologically verified. Almost all patients have immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies to the N protein at the onset of symptoms (Vaheri et al. 2008). Neverthless, in 2-4% of PUUV-cases the seroconversion takes place up to five days from the onset of disease (Vaheri et al. 2008). Most laboratories today use PUUV-IgM enzyme immunoassay based on recombinant N protein (Elgh et al. 1997, Sjölander et al. 1997). In addition, immunochromatographic rapid tests are commercially available (Hujakka et al. 2003). Reverse transcription polymerase chain reaction assay can be useful for the early diagnosis and in hantavirus genotyping (Evander et al. 2007).

Typical laboratory findings include anemia or hemoconcentration due to increased capillary permeability, leukocytosis, thrombocytopenia, elevated liver enzymes, elevated serum creatinine level, hematuria and proteinuria (Vaheri et al. 2003b). Thrombocytopenia and elevated creatinine level are the hallmarks of hantaviral disease. The nadir platelet count (about 100 x 10^9/l) is seen in the febrile phase of illness, 4-5 days from the onset of fever, and normalizes a week after the onset of symptoms (Lähdevirta et al. 1971, Braun et al. 2010, Mustonen et al. 2013). In a Finnish study with 546 PUUV infected patients, thrombocytopenia was severe (< 50x 10^9/l) in 28 % of the subjects, and 90 % were thrombocytopenic (<150x 10^9/l) (Outinen et al. 2016). Also, C-reactive protein (CRP) level is increased (Settegren et al. 1989).

A third of the acute phase patients have abnormal findings in chest radiography, and nearly all patients have lung parenchymal abnormalities in high-resolution computed tomography (Kanerva et al. 1996, Paakkala et al. 2012). The most common pulmonary radiological findings are accumulation of pleural fluid, atelectasis, intralobular and interlobular septal thickening. In addition, lymphadenopathy is detected in a minority of patients (Paakkala et al. 2012). Pulmonary edema is a severe complication of both PUUV and DOBV infections (Vaheri et al. 2013a). Acute-phase cardiac findings, such as electrocardiographic changes appear in 57% of PUUV

patients, which usually include transient T-wave inversions. Left ventricular contraction abnormalities of the heart are also documented in echography (Mäkelä et al. 2009).

2.2.4 Treatment, prevention and prognosis

No specific therapy is currently available in the clinical setting for HFRS. Treatment is mainly supportive, maintaining the fluid and electrocyte balance together with circulatory volume. HFRS patients with severe AKI and fluid retention need hemodialysis treatment. In severe trombocytopenia with bleedings, platetet transfusions are used. In addition, supportive measures for maintaining ventilation function and blood pressure can be needed (Mustonen et al. 2013).

Ribavirin, an antival drug, is yet the only established drug against hantavirus infections with approved *in vitro* and *in vivo* effects against virus replication (Szabó 2017). Administration of ribavirin intravenously may reduce the severity of renal impairment, oliguria and hemorrhagic manifestations, when it is applied early in the onset of symptoms (Szabó 2017). In a recent study with PUUV-HFRS patients, ribavirin was shown to be ineffective in the treatment (Malinin and Platonov 2017). Icatibant, a selective bradykinin (BK) receptor antagonist, has been used successfully in the treatment of two severe PUUV patients with extensive capillary leakage leading to hypotensive shock (Antonen et al. 2013, Laine et al. 2015b).

Furthermore, monoclonal antibodies against HTNV are developed with proven efficacy *in vitro* and *in vivo* studies (Xu et al. 2009, Xu et al. 2002). Small molecule inhibitors targeting $\alpha_v\beta_3$ integrins might have therapeutic potential (Hall et al. 2010). Small molecule inhibitors, targeting vascular endothelial growth factor R2 (VEGF) and Src family kinases signaling responses, are shown to inhibit ANDV-induced endothelial cell permeability (Gorbunova et al. 2011). However, the therapeutic potential of these compounds needs to be further studied.

Vaccines against Hantaan virus and SEOV are in general use in China and in the Rebuplic of Korea, but not outside Asia (Szabó 2017). As to date, no vaccine has been approved for prevention against hantaviruses in Europe and USA. A phase 2 study on human HTNV and PUUV DNA hantavirus vaccine has been ongoing in recent years in the USA (Hooper et al. 2014, Schmaljohn et al. 2014). Some vaccines are mentioned to be under development in pre-clinical stages (Szabó 2017).

PUUV and SAAV usually cause a mild form of disease with mortality up to 0.4 % for PUUV (Hjertqvist et al. 2010, Avšič-Županc et al. 2015). In comparison, HFRS caused by HTNV and DOBV are more severe as the case-fatality rate is as high as 15% and 6-12%, respectively (Avšič-Županc et al. 1999, Vapalahti et al. 2003, Jonsson et al. 2010).

2.3 Pathogenesis of hantavirus infection

2.3.1 Endothelial activation

The endothelial cells of the small capillaries in various organs are the main target in hantavirus infection. The infection of endothelium does not seem to cause direct cytopathologic effects to the infected endothelium *in vitro* studies (Mackow and Gavrilovskaya 2009, Vaheri et al. 2013b). Neverthless, hantavirus antigens are encountered in endothelial cells in HFRS, and in endothelial cells in lung capillaries during HCPS (Cosgriff et al. 1991, Zaki et al. 1995). Vascular endothelial barrier dysfunction with increased capillary permeability and vascular leakage are the characteristics of clinical hantaviral disease (Cosgriff et al. 1991, Gavrilovskaya et al. 2008, Gorbunova et al. 2011). During clinical course, this is represented as hemorrhages, hemoconcentration and hypotension.

Regarding the upregulation of proinflammatory cytokines, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon γ (IFN-γ), all capable of activating the endothelium, have been reported to be elevated in hantavirus disease (Sadeghi et al.

2011, Hepojoki et al. 2014). Proinflammatory cytokines are produced in macrophages or dendritic cells as a response to the recognition of hantaviruses. The change of fenotype of the endothelial cells from anti-adhesive to pro-adhesive bind monocytes through intercellular adhesion molecule 1(ICAM-1) and integrin β2-integrin interaction and platelets through von Willebrand factor (vWF) through αIIbβ3 integrin interaction (Hepojoki et al. 2014). Elevated levels of soluble endothelial cell receptors such as E-selectin, ICAM and tumor necrosis factor receptor (TNFR)-1 are present in acute HFRS (Takala et al. 2000, Han et al. 2010, Kyriakidis and Papa, 2013). Finally, activated macrophages and platelets promote coagulation, fibrinolysis and complement and contact pathway activations along with various mediators involved in immune responses (Hepojoki et al. 2014, Mustonen et al. 2013).

In a Swedish study, levels of endothelial glycocalyx degradation and leukocyte adhesion molecules as indicators of endothelial dysfunction and marker for vascular repair, were elevated and correlated with disease severity of PUUV (Connolly-Anderssen et al. 2014b). Recent study suggests an imbalance between factors contributing to angiogenesis and vascular integrity, namely angiopoietin-1 and its antagonist angiopoietin-2. Thus, PUUV-induced deregulation of angiopoietin levels may contribute to endothelial dysfunction and disease severity (Nushaug et al. 2017).

The lack of appropriate animal model has limited the understanding of hantavirus pathogenesis, although macaque monkeys (*Macaca fascicularis*) infected with wild-type PUUV strains are able to produce a disease resembling human PUUV infection (Klingström et al. 2002, Sironen et al. 2008).

2.3.1.1 Platelet activation

The cellular entry of hantaviruses is mediated by ß3-integrins on platelets, endothelial cells and macrophages (Gavrilovskaya et al. 1999). Hantavirus interacts directly with platelets via GPIIb/IIIa (also known integrin αIIbß3) receptor, which contributes to the binding of quiescent platelets to infected endothelium (Gavrilovskaya et al. 2010),

platelet activation, viral dissemination and induction of endothelial functions. Platelet activation contributes to conformation changes of integrin GPIIb/IIIa receptor, enabling it to bind fibrinogen, vWF and fibronectin required for platelet aggregation (Bennett JS 2005). These events are suggested to contribute to a decreased amount of circulating platelets and increased vascular permeability.

Lowered levels of disintegrin and metalloproteinase with a thrombospondin type 1 domain 13 (ADAMTS13) along with altered platelet ligands of αIIbß3, such as elevated vWF and decreased fibronectin have been reported during acute PUUV-HFRS (Laine et al. 2011). Von Willebrand factor (vWF) is produced in endothelium and megakaryocytes and then carried in platelet α-granules. VWF mediates platelet adhesion and aggregation to the injured endothelium in both primary hemostasis and thrombosis (Sadler JE 1998). Fibrinogen, an acute phase protein and a platelet ligand of αIIbß3, contributes to fibrin-clot formation due to thrombin in vascular and tissue injury. Elevated fibrinogel level correlates to the lowered platelet count in PUUV-HFRS, which further implicates platelet activation and consumption. Enhanced platelet adhesion via vWF and altered platelet ligands thus imply increased platelet adhesion and activation during PUUV-HFRS, which may result to platelet consumption and thrombocytopenia encountered (Laine et al. 2011).

Elevated levels of platelet receptors, soluble P-selectin and glycoprotein VI, a collagen receptor mediating platelet activation at the site of vascular injury where collagen is exposed, have been demonstrated during acute PUUV-HFRS. Furthermore, these *in vivo* markers for platelet activation were especially increased in patients with DIC and thrombosis (Connolly-Andersen et al. 2015).

2.3.1.2 Capillary permeability

Increased vascular permeability in various organs is a typical characteristic of the pathogenesis of hantavirus infection (Cosgriff 1991, Mustonen et al. 2013). Plasma leakage from vasculature into tissues is behind many clinical symptoms and features,

such as hemorrhages, hemoconcentration, hypotension and shock, abdominal symptoms due to retroperitoneal edema and pleural effusion (Cosgriff 1991, Kanerva et al. 1998a). Data suggests that endothelial barrier function is lost because of enhanced permeability instead of direct cellular cytotoxicity or injury of vascular cells. Current knowledge implies that several simultaneously occurring factors are involved in the increased vascular permeability (Hepojoki et al. 2014).

VEGF induces angiogenesis in the endothelium and is able to increase vascular permeability via ß3 integrin signalling (Dvorak 2006). VEGF levels are elevated in hantavirus infection, which is suggested to enhance vascular permeability by degradation of vascular endothelial (VE)-cadherin (Gavrilovskaya et al. 2008, Gorbunova et al. 2010, Li et al. 2012). VE-cadherin, an endothelial adhesion molecule, is important in maintaining cell contact integrity and regulating vascular permeability via VEGF receptor 2, VEGF-R2 (Vestweber 2008). Hantavirus-induced permeability has been inhibited by antibodies against VEGF-R2 (Gavrilovskaya et al. 2008, Gorbunova et al. 2011).

Bradykinin (BK) is an inflammatory peptide of plasma that promotes vascular permeability, edema formation and hypotension. It is produced through the kallikrein-kinin system (Maurer et al. 2011). When hantavirus-infected endothelial cells are incubated with plasma proteins involved in the kallikrein-kinin pathway, the kallikrein-kinin system is activated via factor XII (FXIIa) and BK generation enhanced resulting in an increased vascular permeability *in vitro* (Taylor et al. 2013). Thus, it is suggested that the kallikrein-kinin system activation along with BK contribute to vascular leakage *in vivo*. Few reported cases of severe PUUV-HFRS succesfully treated with BK antagonist, icatibant, further implicate the role of BK in the pathogenesis (Antonen et al. 2013, Laine et al. 2015b).

2.3.2 Role of innate immune response

HFRS is suggested to be a general systemic inflammatory response syndrome, in which the efforts of both innate and adaptive immune system mediate to clear the infection (Mackow and Gavrilovskaya 2009, Vaheri et al. 2013b, Jiang et al. 2016).

2.3.2.1 Cytokines, IL-6 and nitric oxide

Cytokines can explain many symptoms encountered in hantavirus infection. Cytokines are produced in monocytes, macrophages and lymphocytes due to proinflammatory signals, and they take part in the regulation of inflammatory response. Proinflammatory cytokines, tumor necrosis factor (TNF)-α, interleukin-1 (IL-1) and IL-6 are associated with fever, septic shock and induction of acute-phase proteins (Akira et al. 1990).

Increased levels of cytokines have been documented in plasma, urine and kidneys during hantavirus infection (Linderholm et al. 1996, Mäkelä et al. 2004, Saksida et al. 2011, Mustonen et al. 2013). IL-10, IFN- γ and TNF-α are increased both in PUUV- and DOBV-infection in the acute phase, but IL-10 and TNF-α correlate with more severe disease in DOBV infection (Linderholm et al. 1996, Saksida et al. 2011). The imbalance in the production of pro-inflammatory and regulatory cytokines may associate with disease severity (Saksida et al. 2011). During acute PUUV-HFRS elevated IL-6 and TNF-α and low immunosuppressive transforming growth factor-ß1 (TGF-ß1) levels correlate with disease severity characterized by increased creatinine level and low platelet count. In addition, the upregulation of TGF-ß1 in the late phase indicate a protective immunoregulatory role (Sadeghi et al. 2011).

Enhanced nitric oxide (NO) formation associate with elevated TNF-α and with the degree of hypotension in acute PUUV-HFRS (Groeneveld et al. 1995, Linderholm et al. 1996). NO is constitutively formed in the endothelial cells by endothelial nitric oxide synthase (eNOS) and in macrophages and neuthrophils by inducible nitric oxide

synthase (iNOS) mainly due to inflammatory stimuli and cytokines. NO is important both in the inhibition of platelet activation and aggregation and a potent vasodilatator that increases blood flow in vessels. Inadequate NO formation is a significant cause of endothelial dysfunction (Pober and Sessa 2007). NO is reported to have antiviral effects on hantavirus replication cycle in cell culture studies and in mouse models (Klingström et al. 2006). Endothelial cells can also restrict immune response through NO secretion. In addition, cytotoxic T-cells may contribute to the capillary damage via immunopathology, by increased NO and TNF-α in PUUV infection (Groeneveld et al. 1995, Linderholm et al. 1996).

2.3.2.2 Complement system

Complement system is a tightly regulated network of proteins bridging innate and adaptive immunity (Walport 2001, Dunkelberger and Song 2010). It plays an important role in host defense and inflammation and in the clearance of necrotic, apoptotic cells and immunocomplexes. The complement system can be activated by three major pathways: the classical, the alternative and the lectin-dependent. They all converge at complement component C3 which is the main component of the complement in the blood, resulting in the formation of activation products, C3a, C3b, 5a and the end product of the complement cascade, the membrane attack complex (MAC) C5b-9 (Walport 2001). MAC causes the osmotic lysis of the target cells.

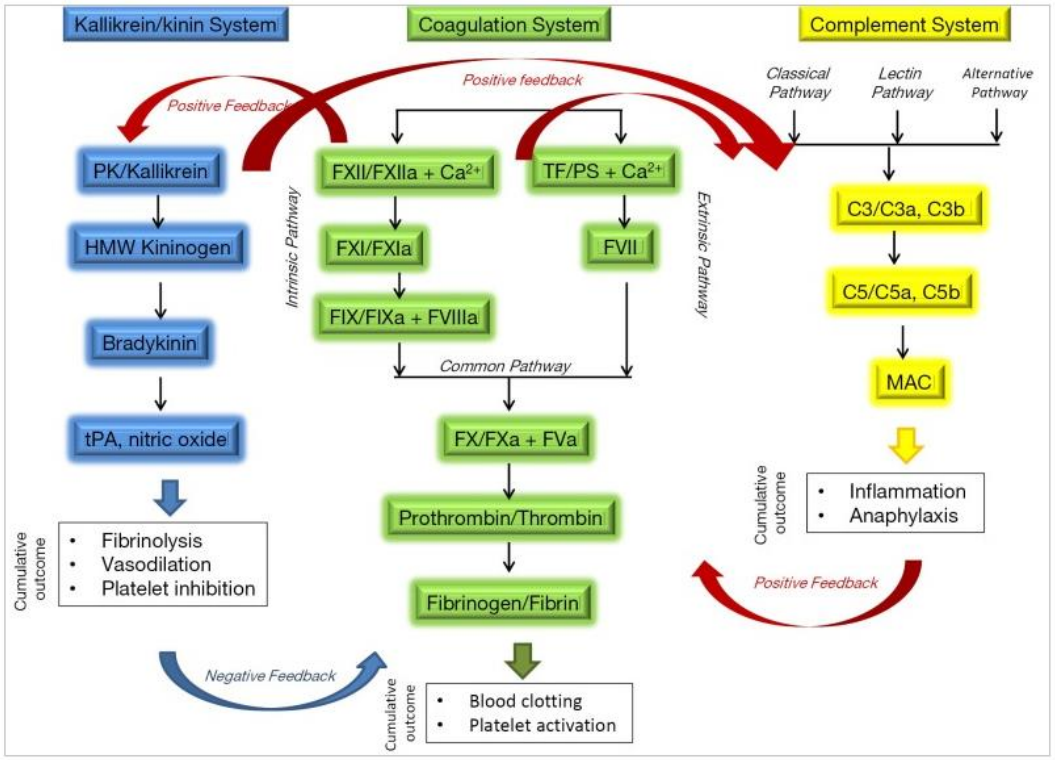
The complement system is known to be linked to the coagulation and fibrinolytic system via multiple interactions through a complex serine protease system (Markiewski et al. 2007). Complement activation can also be triggered beyond the three major routes. Activation of C5 by thrombin was demonstrated in C3 knockout mice, in which C5 convertases cannot be generated (Hubert-Lang et al. 2006). In addition plasmin, kallikrein and FXIIa cleave complement components or fragments. Activated (a) coagulation factor (F) XII is able to activate complement factor CR1 and initiate the activation of classical pathway (Ghebrehiwet et al. 1983). Thrombin, FXIa, Fxa, FIXa

and plasmin are found to cleave C3 into products that display similar molecular weights as the native anaphylatoxins C3a and C5a. These products are also reported to exhibit chemoattraction of human mast cells and neutrophils (Amara et al. 2010). Complex interactions between complement, coagulation and kinin/kallikrein systems are shown in Figure 1.

The complement system is activated in acute HFRS, which is implicated by increased SC5b-9 levels and decreased C3 (Paakkala et al. 2000, Sane et al. 2012). Complement activation of classical pathway associates with more severe clinical disease, although activation through the alternative route has been shown to be more common (Paakkala et al. 2000). Anaphyllotoxins, C3a and C5a, can contribute to the endothelial cell activation and permeability along with directing MAC-mediated vascular injury (Kerr and Richards 2012). Hantavirus infection induces galectin-3 binding protein (Gal-3BP), a glycoprotein reported in chronic viral infections, and it associates with MAC levels. Excessive Gal-3BP formation has been suggested to sensitize the infected cells for complement attack (Hepojoki et al. 2014). Complement attack against glomerular endothelial cells may also contribute to kidney dysfunction in hantavirus infection, as Gal-3BP is found to be produced in glomeruli and tubular epithelium of PUUV-infected macaques (Hepojoki et al. 2014).

SC5b-9 can increase endothelial permeability via ligating ß3-integrin, releasing BK and platelet-activating factor (Tsukada et al. 1995, Bossi et al. 2004). Additionally, the MAC complex can mediate cellular reactions and formation of inflammatory cytokines that are able to alter endothelial function. These observations further link complement activation to the loss of endothelial integrity.

Figure 1. Complex relationship between complement, coagulation and kallikrein/kinin systems. The complement, coagulation and kallikrein/kinin systems are working in concert with each other to provide both positive and negative feedback for hemostasis and inflammation. Positive feedback between coagulation and kininogen/kinin systems occurs through FXII ability to activate prekallikrein (PK) and reciprocal effect of PK on FXII activation. FXIIa activates complement system through activation of C1 component of complement triggering classical pathway of activation. A positive feedback also exists between Kallikrein/kinin and complement system. Inflammation promoted through activation of complement and bradykinin-generating arm of kallikrein/kinin system, in turn, facilitates coagulation through activation of extrinsic coagulation pathway. Certain products of kallikrein/kinin system (e.g., tPA) supply important negative feedback to coagulation system by initiating fibrinolysis and leading to clot dissolution and restoration of hemostasis. Contact activation of complement, FXII and kallikrein pathways is responsible for clotting complications. FXII is a common element providing positive feedback between the three systems. HMW=high molecular weight; tPA=tissue plasminogen activator; TF=tissue factor (CD142); PS=phosphatidylserine; "a" following coagulation factor indicates activated form of the factor; C3 and C5 are complement components 3 and 5, respectively, "a" and "b" following complement components indicate split products of these components; MAC=membrane attack complex, also known as terminal complex. (Dobrovolskaia and McNeil 2015. Copyright 2015. Annals of Translational Medicine)



2.3.2.3 Cellular immune response

T cell activation has been associated with the immunopathology of hantavirus infection. Cytotoxic CD8+ T-lymphocytes are vigorously upregulated at the onset of both HFRS and HCPS (Ennis et al. 1997, Kilpatrick et al. 2004, Lindegren et al. 2011). It is suggested that N-protein is the predominant immunomodulant antigen behind the T cell response (Terajima and Ennis 2011). CD8+ T cells are predominantly found in the kidneys during acute phase of PUUV infection and also in the lungs of fatal HCPS cases (Zaki et al. 1995, Temonen et al. 1996). CD8+ T cell infiltrates colonalizing with viral RNA and antigens at site of tissue injury are found in the kidney tubulointerstitium of cynomolgus macaques (*Macaca fascicularis*) infected with PUUV (Sironen et al. 2008). Altogether, CD8+ T cell response may be associated with the hantavirus pathogenesis and capillary leakage through the overproduction of proinflammatory cytokines or by eliminating virus infected cells and viral clearance (Terajima and Ennis 2011). However, cell death of the hantavirus infected endothelial cells is not obvious (Vapalahti et al. 2003). This supported the finding that hantavirus-infected endothelial cells are found to block cell toxicity of the CD8+ cells and natural killer cells *in vitro* study (Gupta et al. 2013).

CD8+ memory T cells are developed during the convalescent phase of PUUV-HFRS (Tuuminen et al. 2007). T cell memory has been shown to last for several years after PUUV and ANDV infection (Van Epps et al. 2002, Manigold et al. 2010), thus providing a long-lasting immunity after hantavirus infection.

2.3.3 Host genetic factors

Host genetic factors have an impact on the clinical course of a hantavirus infection. Genetic predisposition toward severe HFRS disease is related to human leukocyte antigens (HLA), which are cell surface antigens presenting pathogen-derived antigens to T cells and iniating adaptive immune responses. HLA allelels B8, C4A*Q0 and

DRB1*0302 are associated with a severe form of PUUV infection in Finnish patients (Mustonen et al. 1996).

In patients with HTNV-induced HFRS in China HLA-DRB1*09 and HLA-B*46-DRB1*09 haplotypes were more common than controls (Wang et al. 2009). In Slovenia, patients infected with PUUV had HLA-DRB1*13 haplotype more frequently than patients infected with DOBV. Furthermore, in DOBV infected patients HLA-B*35 was more common than in patients infected with PUUV (Korva et al. 2011). The same HLA type is also reported to correlate with a severe course of HCPS induced by SNV (Kilpatrick et al. 2004).

A cytokine polymorphism at position -308 of high-producing TNFα gene is commonly encountered in hospitalized PUUV-infected patients, and it has been found to associate with clinical severity (Kanerva et al. 1998b). Also a severe disease has been linked to a genotype associated with low TNFα formation (a polymorphism at position -238) (Maes et al. 2006). In addition, polymorphisms in the genes encoding plasminogen activator inhibitor-1 (PAI-1) and platelet membrane glycoprotein 1A have been associated with renal impairment and thrombocytopenia during PUUV-HFRS (Laine et al. 2012).

2.4 Thrombocytopenia in hantavirus infection

2.4.1 Role of platelets in innate immunity and inflammation

Platelets are small anucleate discoid-shaped cellular fragments, 2-3 μm in diameter derived from megakaryocytes in the bone marrow (Richardson et al. 2005). Platelets are under regulation of TPO and exist in circulation for 7-10 days. Platelets are the primary regulators of hemostasis and thrombosis. Platelets also play an important role in modulating and driving innate immunity responses, inflammation, angiogenesis and cancer progression (Mancuso and Santagostino 2017). During their normal life-span platelets diminish in size, thus young platelets are larger in size than older ones. Under

normal conditions senecent platelets are removed from the vasculature by neutrophils and macrophages to be destroyed in the spleen.

The physiological function of platelets is to quickly reach damaged vascular endothelium, aggregate and prevent excessive bleeding by a wound. Stable thrombus formation involves initial contact with platelets and subendothelial matrix of injured vessel walls, followed by adhesion, spreading and aggregation. This initial adhesion includes binding of glycoprotein (GP)Ib-V-IX complex to vWF (Savage et al. 1996) followed by the interaction between GPVI and collagen (Massberg et al. 2003), which activates platelet adhesion receptors, such as GIIb-IIIa, mediating irreversible contact with vWF, and GPIa-IIa. Finally, degranulation of α-granules and dense granules release mediators, such as thromboxane A2 and adenosine diphosphate, which maintain and amplify further platelet aggregation. Thrombus formation is accomplished through fibrinogen-mediated interactions on activated platelet receptors.

Upon vascular inflammation or injury, platelets promote leukocyte recruitement to vessel wall. These adhesive interactions include binding P-selectin, which is released from α-granules and expressed on activated platelets, to P-selectin glycoprotein ligand-1 on neutrophils and macrophages (Spertini et al. 1996). P-selectin is considered a key element in initiating leukocyte rolling and adhesion to platelets and endothelium (Thomas and Storey 2015). Platelets are important in immune surveillance by recognizing danger signal from pathogens and cell damage through the expression of all ten toll-like receptors (TLRs) on its surface or internal compartments. The activation of TLRs triggers hemostatic and inflammatory responses which participate in the host´s defence against bacterial and viral infections, thus bridging thrombosis with infection and immunity. Stimulation of TLR4 may be linked to increased formation of neutrophil extracellular traps (NETs), which aid the clearance of bacteria (Clark et al. 2007).

Platelets are a source of pro-inflammatory chemokines and cytokines and also promote inflammation via release of microparticles. In addition, platelets express FcγRIIa, a Fc receptor that recognize immune complexes and IgG-opsonized cells with high affinity (Mancuso and Santagostino, 2017). Furthermore, platelet expression

of CD40L interacts with leukocyte CD40 and induces monocyte tissue factor (TF) expression and activation of coagulation system (Lindmark et al. 2000). CD40L (also known as CD154), released from α-granules, interacts with CD40 expressed on T-cells, B-cells, dendritic cells and monocytes, thus providing a link between innate and adaptive immune responses. CD40, a receptor and CD40L, a ligand, are important mediators of lymphocyte interactions and antigen presenting cells (Grewal et al. 1998). CD40L expression of platelets further enhances leukocyte recruitment to the endothelium (Henn et al. 1998).

Platelet activation with the expression of P-selectin can initiate complement activation via increasing C3b deposition, C3a generation and C5b-9 production (Del Conde et al. 2005). Complement activation occurs in an amplificatory manner, and is mediated by platelet release of chondroitin sulfate (Hamad et al. 2008). Additionally, platelet microparticles that contain complement components such as C1q and C5b-9, take part in the complement activation and microbiological clearance (Yin et al. 2008).

2.4.2 Mechanisms of virus-induced thrombocytopenia

Thrombocytopenia is caused by either decreased platelet production or increased platelet destruction. Viruses can use different mechanisms to decrease the level of circulating platelets. Platelet production can be decreased by infection of megakaryocytes that leads to apoptosis of megakaryocytes, reduced maturation and ploidy of megakaryocytes or decreased expression of TPO receptor (c-Mpl). Human immunodeficiency virus (HIV), cytomegalovirus (CMV) and hepatitis C virus (HCV) are known to replicate and infect megakaryocytes (Chelucci et al. 1998, Li et al. 1999, Crapnell et al. 2000). Some viruses, such as HIV, CMV and dengue, are able to infect hematopoietic stem cells resulting in altered cytokine production and decreased progenitor cells, thus suppressing hematopoiesis (Kolb-Mäurer and Goebel 2003). Viruses may directly interfere with TPO production as shown for HCV (Afdhal et al. 2008).

Interaction between platelets and viruses can lead to enhanced platelet destruction via a variety of receptors, mainly mediated by integrins, lectins and TLR (Assinger 2014). Hantavirus and also hantavirus-infected endothelial cells interact with platelets via receptor GPIIb/IIIa (also known as integrin αIIbß3), which results in platelet activation and possibly clearance. B-lymphocyte production of antibodies against HCV, HIV, CMV, Ebstein Barr virus, hantavirus, varizella zoster virus and herpes virus has been suggested to intervene with platelet survival (Goeijenbier et al. 2012). Furthermore, disturbed portal vein pressure and enhanced sequestration in enlarged spleen, as in HCV infection, may contribute to thrombocytopenia (Assinger 2014).

2.4.3 Thrombocytopenia and associated predictive markers for disease severity

Decreased platelet count may contribute to increased propability of bleedings. Recent studies have elucidated the multiple mechanisms of thrombocytopenia in hantavirus infection. The interaction of platelets with infected endothelium is considered to be important (Gavrilovskaya et al. 2010, Vaheri et al. 2013b). Findings of altered platelet ligands suggest increased platelet activation and consumption due to coagulation activation during PUUV infection (Laine et al. 2011, Connolly-Andersen et al. 2015). Increased mean platelet volume and immature platelet fraction together with elevated TPO level indicate active thrombopoiesis in the bone marrow (Connolly-Andersen et al. 2015, Laine et al. 2015a, Laine et al. 2016). This is further supported by bone marrow examinations that have shown increased amount and size of megakaryocytes during HTNV and PUUV infections (Lähdevirta 1971, Lee 1987). In addition, platelet aggregation is impaired especially when induced by thrombin, and associates with a decreased platelet count, although defective aggregation was noted also among patients with a nearly normal platelet count. However, platelet adhesion to collagen surface is intact when assessed by a platelet function test, PFA-100® (Laine et al. 2015a).

Decreased platelet count during hantavirus infection has been linked to various predictive markers of severe disease. Increased levels of circulating plasma cell-free

deoxyribonucleic acid (cf-DNA) have been reported to associate with thrombocytopenia and leukocytosis during acute PUUV-HFRS (Outinen et al. 2012). Cell free DNA originates from apoptotic and necrotic cells, and it is considered to indicate the amount of cellular damage (Jahr et al. 2001). However, the urinary excretion of cf-DNA is not increased and does not correlate with the severity of AKI. Elevated levels of cf-DNA possibly reflect the apoptosis occurring in the acute phase as the levels correlate with cf-DNA band intensity in quantitative analysis (Outinen et al. 2012).

IL-6 is a proinflammatory cytokine responsible for acute phase reactants, inflammatory responses by activating T cells and promoting B cell differentiation (Kishimoto et al. 1995). In PUUV-HFRS, increased plasma IL-6 levels have been associated with both severe AKI and more severe thrombocytopenia (Linderholm et al. 1996, Takala et al. 2000, Outinen et al. 2010).

Pentraxin-3 (PTX-3), an acute phase protein, is generated at the site of inflammation in various cells and tissues, mainly by dendritic cells and macrophages due to cytokines, IL-1ß, TNF-α and TLR activation (Bottazzi et al. 2009). PTX-3 recognizes a diverse range of pathogens: bacteria, viruses and fungi, modulates complement activity by binding C1q and facilitates pathogen recognition by macrophages and dendritic cells (Mantovani et. al 2008). In additon, PTX-3 interacts with factor H which activates the alternative pathway of the complement (Deban et al. 2008). High PTX-3 levels during acute PUUV-HFRS associate with severe outcome of the disease, as evaluated by the severity of AKI, thrombocytopenia and length of hospitalization (Outinen et al. 2012). PTX-3 has considered to be a part of the pathogenesis because of the crosslinkage of coagulation and complement activation (Mustonen et al. 2013).

2.5 Activation of coagulation system in hantavirus infection

2.5.1 Coagulation activation

TF is the major activator of coagulation cascade during viral infection. In response to vascular injury, inflammatory cytokines such as TNF-α and IL-1ß, or other stimuli, TF is highly expressed in adventitial fibroblasts, smooth muscle cells, pericytes in the subendothelial tissue and by circulating monocytes. TF expression is sustained by proinflammatory cytokines, chemokines and procoagulative microparticles (MPs), which are phospholipid membrane vesicles shed from various cell types. MPs derived from platelets and erythrocytes are able to activate thrombin generation in factor XII-dependent manner (Van Der Meijden et al. 2012). TF exposure into blood allows it to form a complex with activated coagulation factor VII (FVIIa). This TF/FVIIa complex is essential in triggering the coagulation cascade, which ultimately leads to the formation of thrombin.

A study with PUUV-infected human umbilical vein endothelial cells showed increased TF expression in endothelium (Goeijenbier et al. 2015). Coagulation can be induced by both TF (i.e. extrinsic pathway) and contact system, also known as plasma kinin-kallikrein system (i.e. intrinsic) pathways. Elevated plasma kallikrein in HFRS patients suggests the activation of contact pathway (Guang et al. 1989). Incubation of plasma protein with hantavirus-infected endothelial cells has shown to result in cleavage of high molecular weight kininogen, elevation in enzymatic activities of FXIIa and BK liberation, thus indicating plasma kallikrein-kinin system activation (Taylor et al. 2013). The activation of the contact system can be both procoagulative via the activation of intrinsic coagulation pathway and proinflammatory via the kinin-kallikrein system activation leading to BK (Wu 2015).

An early study of coagulopathy with HTNV-induced HFRS patients showed a prolongation of bleeding time together with decreased platelet count and survival (Lee et al. 1987). In the acute phase, prothrombin time (PT) and partial thromboplastin time

were prolonged. The levels of activated coagulation factors II, V, VIII, IX and X were also lowered, but factor VIII antigen level and VWF ristocetin cofactor activity were not changed. The fibrinogen level was diminished in 20% of patients during hypotensive and oliguric phases. Thrombin time (TT) was prolonged, and increased amounts of fibrin degradation products were noted. When studied with thromboelastogram, reaction and clotting times were prolonged due to lowered coagulation factors, and maximum amplitude was decreased, suggested to reflect thrombocytopenia in the acute phase (Lee et al. 1987).

Studies on PUUV-HFRS suggest that both coagulation and fibrinolysis are active in the acute phase (Lähdevirta 1989, Laine et al. 2010, Laine et al. 2011). Increased prothrombin fragments (F1+2) were reported in 18 out 19 patients with acute PUUV-HFRS, indicating increased *in vivo* thrombin formation (Laine et al. 2010). Diminished levels of physiologic anticoagulants, antithrombin (AT), protein S free antigen (PS), protein C (PC) were found to correlate with thrombocytopenia. Shortened TT was observed (Laine et al. 2010).

2.5.2 Fibrinolysis

The breakdown of fibrin blood clots is driven by plasmin, which is the key enzyme of the fibrinolytic system. Formed thrombin activates tissue plasminogen activator (tPA) and urokinase type-plasminogen activator (uPA), which cleave plasminogen on the surface of the fibrin clot or on cell surfaces to generate plasmin (Chapin and Hajjar, 2015). Endothelial cells produce tPA, whereas uPA is synthesized by monocytes, macrophages and urinary epithelium. Plasminogen activator inhibitor-1 (PAI-1), mostly derived from activated platelets, inhibits plasminogen activators, tPA and uPA to prevent excessive fibrinolysis and to warrant clot stabilisation (Sprengers and Kluft, 1987). Fibrin degradation products, among D-dimer fragments, are released as plasmin cleaves thrombus.

Fibrinolysis is increased during acute PUUV-HFRS. In a study with PUUV-HFRS infected patients a 24-fold increase in acute phase D-dimer levels was noted, which is suggestive of enhanced fibrinolysis (Laine et al. 2010). Also, tPA is strongly upregulated acutely, but PAI-1 levels remain unaltered. This finding is suggestive of increased tPA activity. Furthermore, interferons induced tPA expression directly through signal transducer and activator of transcription 1 (STAT1). Elevated tPA levels associate with disease severity, length of hospital stay, weight gain, the minimum platelet count, leukocytosis, high levels of terminal complement complex, IL-6 and hematocrit level (Strandin et al. 2016). A recent study with PUUV infected human umbilical vein endothelial cells demonstrated that hantaviruses are also able to inrease PAI-1 levels in the acute phase (Goeijenbier et al. 2015).

In a study with SNV caused HCPS, patients markedly increased PAI-1 levels were found to correlate with fatal disease (Bondu et al. 2015). When SNV infected macaques developing HCPS like disease were studied, no fibrin degradation products were found (Safronetz et al. 2014). Altogether, this implies decreased fibrinolysis in severe HCPS.

2.5.3 Disseminated intravascular coagulopathy

Signs of DIC are reported during acute hantavirus infection (Lee 1987, Settegren et al. 1989, Cosgriff 1991, Valtonen et al. 1995). However, the applied diagnostic criteria for DIC vary in different studies. Laboratory abnormalities in this consumption coagulopathy involve prolonged activated thromboplastin time (APTT) and PT/international normalized ratio (INR), decreased levels of platelet count and fibrinogen and increased amount of fibrin degradation products, D-dimer. The excessive activation of coagulation includes thrombin generation possibly leading to microvascular thrombosis, when overcoming the anticoagulant system. The consumption of coagulation factors and platelets may lead to hemorrhages seen in DIC (Boral et al. 2016).

In HTNV caused HFRS, DIC could be diagnosed in all patients on the fourth day of illness assessed by early Colman`s criteria (Lee 1987, Colman et al. 1972). DIC was common in the early days of clinical symptoms. The abnormalities in coagulation profile included decreased platelet count, prolonged bleeding time, PT and partial thromboplastin time, reduced levels of coagulation factors, increased fibrinolytic activity, presence of plasma procoagulant activity and fibrin thrombi in autopsy (Lee 1987).

As for PUUV, DIC was diagnosed according to the modified scoring system of the International Society of Thrombosis and Haemostasis (ISTH), in 28% of patients (Sundberg et al. 2011). DIC was associated with more severe disease. Similar finding were reported in a Finnish study where DIC could be diagnosed in five of the 19 hospital-treated patients according to the ISTH criteria (Laine et al. 2010). However, no associations were found between positive DIC score and elevated levels of D-dimer and F1+2. Also, the score was not predictive of the clinical outcome of PUUV infection (Laine et al. 2010).

3 AIMS OF THE STUDY

The aims of the present study were:

1. To assess whether the spleen is enlarged in an acute PUUV infection and its associations with the platelet count and disease severity. (study I)

2. To evaluate the associations of disease severity markers PTX-3, cf-DNA, SC5b-9 and IL-6 with blood coagulation and fibrinolysis in PUUV infection. (study II)

3. To study thrombin generation by CAT® assay and relate the findings with blood coagulation, fibrinolysis and clinical course of PUUV infection. (study III)

4. To evaluate associations between genetic polymorphisms involved in nitric oxide synthases and the clinical course of PUUV infection. (study IV)

4 PATIENTS AND METHODS

4.1 Patients

The population in studies I-IV consisted of prospectively collected, consecutive patients hospitalized in Tampere University Hospital, Finland due to serologically confirmed acute PUUV infection. Studies I and IV were conducted in Tampere University Hospital and the University of Tampere Faculty of Medicine and Life Sciences. Studies II-III were carried out in Tampere University Hospital, the University of Tampere Faculty of Medicine and Life Sciences, the University of Helsinki Haartman Institute and HUSLAB Laboratory Services of Helsinki University Central Hospital.

Study I involved twenty patients who participated in a previous study determining renal MRI findings between October 2000 and October 2002 (Paakkala et al. 2005). Seven patients had one or more of the following chronic diseases: Crohn`s disease, essential hypertension, coronary artery disease, hypercholesterolemia, type II diabetes, depression and epilepsy. None of the patients had chronic kidney disease, liver disease or hematologic malignancy.

Study II consisted of 19 patients selected from those who participated in a previous larger study based on the availability of laboratory samples. Patients were treated in Pirkanmaa University Hospital during the period from September 2000 to December 2002. Regarding medical history, two patients had neurological diseases, and one a chronic inflammatory bowel disease treated with mesalazine medication. Other chronic diseases were: dyslipidemia (n = 2), coronary heart disease (n = 1), arterial hypertension (n = 1) and paroxysmal atrial fibrillation (n = 1). Two patients used an antiplatelet drug, acetylsalic acid.

Study III involved a cohort of 27 patients hospitalized between October 2010 and February 2014. None of the subjects used anticoagulant or immunosuppressive therapy. Concomitant diseases included arterial hypertension (n=4), diabetes mellitus (n=3), coronary artery disease (n=2), hypercholesterolemia (n=2) and obstructive sleep apnea (n=2). Other co-morbidities were chronic obstructive pulmonary disease and celiac disease (n=1 for each). None of the subjects used anticoagulant or immunosuppressive therapy. Two of the patients used an antiplatelet drug, acetylsalic acid.

Study IV consisted of 172 prospectively collected patients treated during the period from September 1997 to February 2009. Concomitant diseases of patients were: arterial hypertension (n=12), dyslipidemia (n=7), coronary artery disease (n=5), bronchial asthma (n=6), atrial fibrillation (n=3) and rheumatoid arthritis (n=3). There were also patients with celiac disease, inflammatory bowel disease, valvular heart disease or neurological disease (n=2 for each).The age and sex distribution of the patients involved in studies I-IV are shown in Table 2.

Table 2. Distribution of age and sex of the patients in studies I-IV.

	Study I	Study II	Study III	Study IV
Patients (n)	20	19	27	172
Male/female	17/3	17/2	17/10	119/53
Age (years)[a]	36 (17-72)	38 (30-67)	50 (21-67)	40 (15-74)

[a] median (range)

4.2 Study designs

In study I, abdominal MRI examinations were performed on admission to the hospital, mean 7 (range 4-12) days from onset of fever, in the acute phase of PUUV infection, and repeated on mean 196 (range 151–267) days from onset of fever, in the control phase. The mean of these two measurements was defined as an acute-phase and control-phase spleen and renal length. The calculations of both acute- and control-phase imaging of spleen and renal lengths were made twice retrospectively, at an interval of two weeks for intraobserver agreement calculation. The radiologist (Antti Paakkala) was unaware of the patients` clinical data and their initial measurements. The patients were used as their own controls by comparing the mean of spleen length in the primary and repeat MRI studies. The laboratory variables, blood count, serum creatinine and CRP values were measured by standard methods mostly daily according to clinical needs.

In study II, three blood samples for each patient were taken during acute phase of illness for the assessment of coagulation and fibrinolysis. The first sample was obtained 2-9 (median 6) days from the onset of fever, the second 3-10 (median 7) days and the third sample 6-13 (median 10) days from the onset of fever. Citrate-anticoagulated (109 mM sodium citrate) samples were centrifuged at 1500 g for 20 min and separated plasma samples were frozen at -70 °C. The defrozen samples were recentrifuged at 2500 g for 15 min prior to analysis. Blood samples for the analysis of plasma SC5b-9 and C3 were taken four (range 2-6) times, the first sample 3-7 (median 6) days from the onset of fever. The blood samples for SC5b-9 and C3/4 analysis were stored at –70 °C and –20 °C, respectively. Samples for the measurement of PTX3 (median 4 times, range 3-5) and cf-DNA (median 3 times, range 3-5) were all obtained up to five days from admission to the hospital. The samples of both PTX3 and cf-DNA were stored at -70 °C before analysis. The blood samples for IL-6 were drawn on three consecutive mornings beginning on the first morning of hospitalization.

In study III, acute phase blood samples for CAT® analysis were drawn median 7 days (range 4-12 days) from the onset of fever. Control samples of 23 patients were

obtained median 43 days (range 38-76 days) from the onset of fever. Blood samples were collected into sodium citrate anticoagulant (3.2%; 109 mM) tubes and centrifuged (at 2500 g for 15 min). The PPP was separated within 2 hours and stored at -80 °C before analysis.

In study IV, DNA for genotyping was extracted from whole blood using a commercially available kit (QIAGEN Inc., Hilden, Germany).

In studies I-III the control phase MRI examination and blood samples taken were used as controls. In study IV, the data on allele frequencies of eNOS and iNOS in the general population was not available. The purpose of the study was to evaluate the severity of the disease in hospitalized patients instead of prevalence.

4.3 Methods

4.3.1 Diagnosis of PUUV infection

The serological diagnosis was verified and based on an IgM-capture enzyme immunoassay and PUUV Sotkamo strain full-length N protein expressed by the baculovirus system in Sf9 insect cells (Vapalahti et al. 1996). The assay has showed optimal sensitivity and specificity in comparison with other protocols (Sjölander et al. 1997).

4.3.2 Laboratory and clinical variables

During the hospital stay the basic laboratory tests, such as complete blood count, plasma creatinine level and CRP level were taken according to clinical indication. Laboratory tests were determined at the Laboratory Centre of the Pirkanmaa Hospital District using standard methods.

The following clinical variables were recorded: number of days from the onset of illness (i.e. fever) before the first blood test was obtained, the length of hospitalization (days), clinical diagnosis of hypotensive shock (yes/no), need for transient hemodialysis treatment (yes/no), the lowest systolic and diastolic blood pressure (mmHg), the lowest daily urinary output (ml) and change in weight (kg). The latter clinical variable is considered to reflect the fluid accumulation in the body during the oliguric phase of the disease. The bleeding symptoms and thrombotic events were also recorded in studies II-III.

4.3.3 Magnetic resonance imaging of spleen (study I)

In study I, MRI was performed using the scanner Signa Horizon Echospeed LX (General Electric, Milwaukee, WI) operating at 1.5 T, using torso phase array coil. The maximum spleen length was calculated by the same radiologist (A.P.) on coronal T2 fast spin echo fat suppression sequence with advantage Windows 4.3 workstation (General Electric Medical System, Paris).

Renal length was calculated on coronal T1 SE FS contrast-enhanced sequence and parenchymal thickness on coronal T2 FSE FS sequence with Advantage Windows 4.0 workstation (General Electric Medical Systems, Paris). When measuring the renal length, first both kidneys were reformed on the oblique coronal plane, and then the maximal length was measured.

4.3.4 Markers of coagulation and fibrinolysis (studies II-III)

Laboratory analysis involved APTT (Actin FSL, Siemens Healthcare Diagnostics, Marburg, Germany), PS (automated latex ligand immunoassay by Instrumentation Laboratory, Lexington, Massachusetts, USA) and AT and PC activities (both measured by chromogenic assays, BerichromAntithrombin III and BerichromProtein C, respectively, Siemens Healthcare Diagnostics) were all carried out using automated

coagulation instrument (BCS XP, Siemens Healthcare System, Marburg, Germany). D-dimer (an immunoturbidimetric assay Tina-quant D-Dimer, Roche Diagnostics, Mannheim, Germany) and prothrombin fragments (F1+2, a monoclonal enzyme immunoassay Enzygnost F1+2, Siemens Healthcare Diagnostics) were determined. The activity of ADAMTS13 was determined by immunochemical detection of its proteolytic target, VWF (Technozym Elisa; Technoclone, Vienna, Austria).

The reference values for APTT were 23-33 s, PS 66-158% for men and 50-177% for women, AT 84-108%, PC 74-141%, TT 17-25 s, fibrinogen 1.7-4.0 g/l, D-dimer ≤ 0.5 mg/l, F1+2 69-229 pmol/l and ADAMTS13 activity 40-130%. Laboratory analyses were made in the Laboratory of Helsinki University Central Hospital.

4.3.5 Analysis of PTX-3, cf-DNA, IL-6 and complement system (study II)

Plasma PTX3 concentration was assessed by an immunoassay (Quantikine; R&D Systems Inc., Minneapolis, Minnesota, USA). The plasma cf-DNA level was measured using a fluorescence-based (a DNA-intercalating dye) Quant-iT DNA High-Sensitivity Assay kit and a Qubit fluorometer (Invitrogen, Carlsbad, California, USA). Plasma samples of cf-DNA were analysed in duplicate and the mean of the two values was considered as the final value. The assessed intra-day variation coefficients at the mean plasma cf-DNA levels of 0.673 μg/ml, 0.876 μg/ml and 1.59 μg/ml were 4.2%, 1.0% and 4.1%, respectively. Plasma IL-6 concentrations were measured by an enzyme-linked immunosorbent assay (PeliKine Compact human IL-6 kit; Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). The reference value was 0.4 pg/ml for IL-6. Complement analyses were performed at Haartman Institute and in the Laboratory of Helsinki University Central Hospital. The plasma C3 levels were analyzed by nephelometry (Dade Behring, Marburg, Germany) and SC5b-9 by an enzyme-linked immunosorbent assay (Quidel, San Diego, California, USA).

4.3.6 Thrombin generation (study III)

In study III, thrombin generation was measured by CAT® (Diagnostica Stago, Thrombinoscope BV, Maastricht Netherlands) with the Stago platelet poor plasma (PPP) reagent (tissue factor 5 pM and phospholipids 4 μM) without corn-trypsin inhibitor. The lag time of the initiation of TG (LT, min), the endogenous thrombin potential (ETP; the area under the curve; nM thrombin x time), peak (maximum thrombin concentration, nM) and time to peak (tt Peak, min) were measured and recorded according to the manufacturer's instructions. Blood samples were collected into sodium citrate anticoagulant (3.2%; 109 mM) tubes according to the local sampling protocol as part of hospital routine, and centrifuged (at 2500 g for 15 min). PPP was separated within 2 hours and stored at -80 °C before analysis. CAT® analyses, plasma measurements of fibrinogen, F1+2 and D-dimer were performed in Clinical Chemistry coagulation laboratory (HUSLAB Laboratory Services) in the Helsinki University Central Hospital.

4.3.7 Genotyping (study IV)

The gene polymorphisms of eNOS G894T (Glu298Asp, rs1799983) and iNOS G2087A (Ser608Leu, rs2297518) were genotyped with TaqMan® SNP Genotyping Assay (Life Technologies Ltd, Carlsbad, CA, USA) under standard conditions using the ABI Prism 7900HT Sequence Detection System (Taqman, Applied Biosystems, Foster City, CA, USA). Reaction volume used was 5 μl and it was prepared with TaqMan Genotyping MasterMix (Life Technologies Ltd, Carlsbad, CA, USA). Amplification data was analyzed with SDS 2.2 software (Taqman, Applied Biosystems, Foster City, CA, USA). The distributions of all SNPs did not deviate from the Hardy–Weinberg equation.

4.3.8 Definition of acute kidney injury (study IV)

In study IV, AKI was determined according to Kidney Disease: Improving Global Outcomes (KDIGO) criteria as an increase in plasma creatinine ≥1.5 times baseline, which was presumed to have occurred within the prior seven days. The upper limits of the reference values for plasma creatinine (women 90 μmol/l and men 100 μmol/l) were taken as baseline levels. In women, plasma creatinine level ≥135 μmol/l and in men ≥ 150 μmol/l was considered as AKI.

4.3.9 Statistical analyses

In studies I-IV, the highest and lowest values of continuous variables measured in the acute phase were designated as maximum or minimum values. In studies II-III, the highest values of APTT, F1+2, D-dimer, IL-6, SC5b-9, cf-DNA and PTX3 and the lowest values of platelet count, TT, fibrinogen, AT, PC, PS, ADAMTS13 and F1+2 were used. In study II, the highest value of SC5b-9 was considered to represent the peak of complement activation, and the lowest values of C3 was chosen to reflect the consumption of complement component.

Medians and ranges were given for continuous variables and numbers for categorical variables. In studies I and III, Wilcoxon-signed rank test was used for pairwise comparisons to analyze the changes between the acute and control phase. Correlations for continuous variables were calculated by the Spearman`s rank correlation test.

In study IV, the allele frequencies were calculated, and the patients were grouped into carriers (including both homozygotes and heterozygotes) and non-carriers of specific alleles. Differences in the clinical severity of PUUV infection between groups were tested using the Mann-Whitney U-test or Kruskal-Wallis test for numerical data and χ^2-test or Fisher`s exact test for categorical data, as appropriate.

In all studies the statistical significance was considered at 0.05 (2-tailed). Statistical analyses were performed using IBM SPSS Statistics for Windows software (version 20 in study I, version 14.0 in study II, version 22.0 in study III and version 21 in study IV).

4.4 Ethical considerations

All patients were recruited and enrolled after providing a written informed consent. Informed verbal consent was obtained from the guardians of the minors (study IV). The Ethics Committee of Tampere University Hospital approved all study designs. Studies were conducted according to the principles expressed in the Declaration of Helsinki.

5 RESULTS

5.1 Characteristics of study population (I-IV)

The clinical and common laboratory findings of the participating patients in studies I-IV are presented in Table 3. In studies III and IV two (8%) and three (2%) patients, respectively, were in clinical shock on admission to hospital. In study II, minor bleedings were observed, as two patients had epistaxis and one conjuntival bleeding. In study III, mild bleedings were reported in eight patients, including nasal and conjunctival hemorrhages, petechiae, hemoptysis and melena. No thrombotic events were encountered in any studies. All patients recovered.

Table 3. Laboratory and clinical findings of patients in studies I-IV.

	Study I	Study II	Study III	Study IV
$Creatinine_{max}$ (µmol/l)	294(78-950)	321(74-1285)	268(71-983)	185(51-1499)
$Platelet\ count_{min}$ ($x10^9$/l)	70(14-192)	75(13-238)	60(5-150)	62(3-238)
$Leukocyte\ count_{max}$ ($x10^9$/l)	11.4(8-19.3)	11.8(7.3-23.2)	10.7(4-45)	10(3.9-31)
CRP_{max} (mg/ml)	96(13-269)	61(6-198)	79(21-244)	75(11-269)
$Hematocrit_{min}$	0.36(0.25-0.43)	0.36(0.28-0.45)	0.36(0.26-0.41)	0.36(0.25-0.46)
$Hematocrit_{max}$	0.45(0.29-0.55)		0.43(0.37-0.60)	0.44(0.33-0.60)
Hospital stay (days)	7(3-15)	7(3-15)	7(3-22)	6(2-15)
Change in weight (kg)*	2.2(0-8.9)	3.2(0.2-12.0)	3.8(0.5-11.3)	2.1(0-12.0)

The values are presented as median (range). Abbreviations: min=minimum, max=maximum, CRP=C-reactive protein. *Change in weight reflects fluid accumulation of body during the oliguric phase. Reference values: hematocrit 0.35-0.50 for men and 0.35-0.46 for women, platelet count 150-360 $x10^9$/l, leukocyte count 3.4-8.2 $x10^9$/l, CRP < 10 mg/ml, creatinine < 105 µmol/l for men and < 95 µmol/l for women.

5.2 Spleen length, platelet count and outcome of PUUV infection (I)

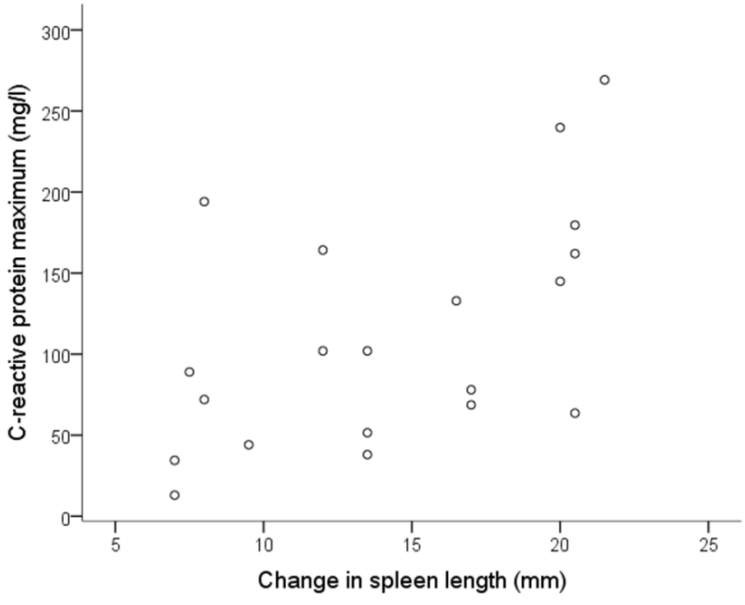
In study I, increased spleen length assessed by MRI was noted in all patients during acute PUUV infection compared to control phase imaging (median 129 mm, range 104-154 mm, vs. median 111 mm, range 92-140 mm, $p < 0.001$; Figure 1 in the original publication). The median change was 15 mm (range 7-22 mm), when the primary and repeat MRI studies were compared.

In 90 % (n=18) of the patients platelet count was below the lover limit of the reference value (< 150 x 10^9/l) during hospital stay (median 7 days, range 3-15 days). Five patients (25%) had severely lowered platelet count 50 x 10^9/l or less. The lowest platelet count measured during hospital stay did not associate with enlarged spleen ($r=0.171$, $p=0.472$, Figure 2c). The change of spleen length correlated positively with the maximum CRP level and inversely with the highest leukocyte count ($r=0.513$, $p=0.021$ and $r=-0.471$, $p=0.036$, respectively; Figures 2a-b).

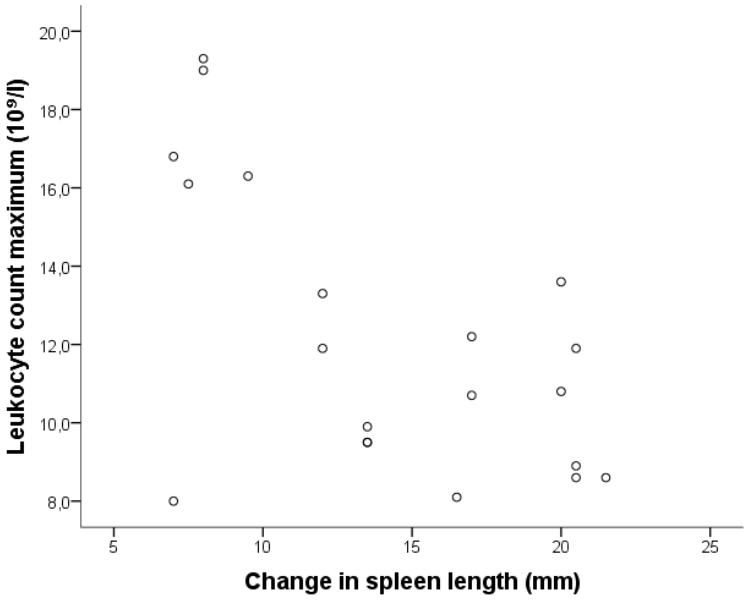
When compared with variables reflecting disease severity, maximum plasma creatinine, the length of hospital duration and change in weight, no correlations were found ($r=-0.306$, $p=0.189$; $r=0.034$, $p=0.887$ and $r=-0.011$, $p=0.965$, respectively). Furthermore, the change in spleen length did not correlate with the maximum or minimum hematocrit ($r=-0.034$, $p=0.887$ and $r=0.208$, $p=0.378$, respectively). Spleen length was associated with renal length (the mean length of right and left kidney) in the acute phase MRI study ($r=0.448$, $p=0.048$). No association was found between the change in the spleen and renal lengths (the mean length of the right and left kidney).

Figure 2. Correlations between the change in spleen length and a) the highest C-reactive protein level, b) the highest leukocyte count and c) the minimum platelet count in 20 patients with acute Puumala hantavirus infection.

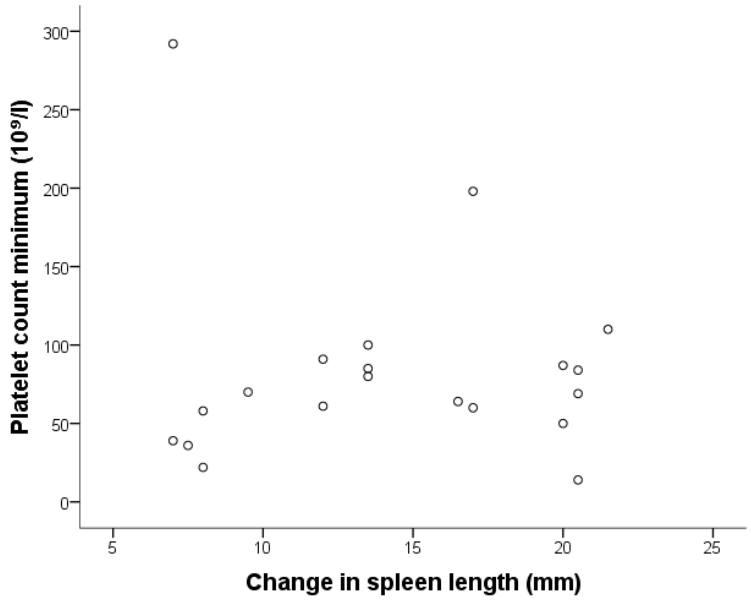
a)



b)



c)



5.2 Associations between coagulation, fibrinolysis, pentraxin-3, cell-free DNA, interleukin-6 and complement system (II)

The laboratory markers for coagulation, fibrinolysis and endothelial cell activation in 19 patients during acute and recovery phase of PUUV infection have been described earlier (Laine et al. 2010, Laine et al. 2011). The values of common laboratory tests and the predictive markers of the studied patients are presented in Table 1 in the original publication. Numerous associations between coagulation variables and predictive markers could be established. The most notable finding in study II was the link between plasma PTX3 and different variables reflecting activation of endothelium and coagulation (Table 4). In addition, low level of the natural anticoagulant AT was associated with elevated levels of predictive markers for disease severity of PUUV infection (Table 5).

Table 4. Associations of plasma pentraxin-3 levels and coagulation markers in 19 patients with acute PUUV infection.

	Plasma pentraxin-3	
	r	**P-value**
Prothrombin fragments $F1+2_{min}$	0.46	0.05
$Fibrinogen_{min}$	-0.70	<0.001
$Antithrombin_{min}$	-0.74	<0.001
Protein C $activity_{min}$	-0.77	<0.001
Protein S $activity_{min}$	-0.81	<0.001
$ADAMTS13_{min}$	-0.48	0.04

Abbreviations: max=maximum, min=minimum, ADAMTS13=a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13.

Table 5. Associations of antithrombin with predictive markers of disease severity in 19 patients with Puumala hantavirus infection.

	Plasma antithrombin	
	r	**P-value**
$C3_{min}$	0.76	<0.001
Plasma $pentraxin\text{-}3_{max}$	-0.74	<0.001
$Interleukin\text{-}6_{max}$	-0.56	0.01
Cell free DNA_{max}	-0.47	0.04

Abbreviations: C3=complement protein C3; min=minimum; max=maximum.

Plasma cf-DNA correlated with APTT, a screening test for the evaluation of intrinsic coagulation pathway, and prothrombin fragments F1+2 (r=0.47, p=0.04 and r=0.49, p=0.03, respectively). In addition, significant associations between cf-DNA

values and complement components reflecting complement activation, C3 and SC5b-9 were found (r=-0.55, p=0.02 and r=0.61, p=0.01, respectively). The levels of plasma C3 correlated with APTT, negatively with natural anticoagulants, PC and PS activities (r=-0.70, p=0.003; r=0.53, p=0.03 and r=0.64, p=0.004, respectively). Increased D-dimer showed no associations with the studied predictive markers.

5.3 Thrombin generation by CAT® (III)

In study III, thrombin generation measured in platelet poor plasma (PPP) of 27 patients by CAT® showed decreased endogenous thrombin potential (ETP) along with decreased peak thrombin concentration during acute phase of PUUV infection (Table 6 and Figure 3a-b). Lag time (LT) and time to reach the peak (tt peak) thrombin concentration were prolonged compared to the CAT® assay carried out at full recovery (Table 6 and Figure 3c-d).

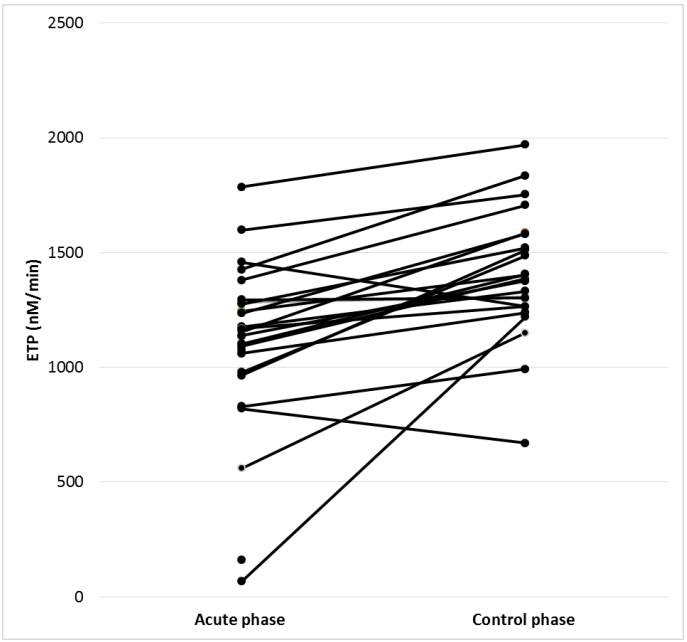
Table 6. Variables reflecting thrombin generation measured by CAT® in the acute phase (n=27) and in the control phase (n=23) of Puumala hantavirus infection.

CAT® parametrs	Acute phase median (range)	Control phase median (range)	p-value
Lag Time (min)	3.8 (2.1-7.7)	2.9 (2.0-4.1)	<0.001
ETP (nM/min)	1154 (67-1785)	1385 (670-1970)	<0.001
Peak (nM)	204 (5.6-293)	243 (106-331)	0.008
Time to Peak (min)	7.3 (4.8-14.9)	5.9 (4.3-9.8)	0.012

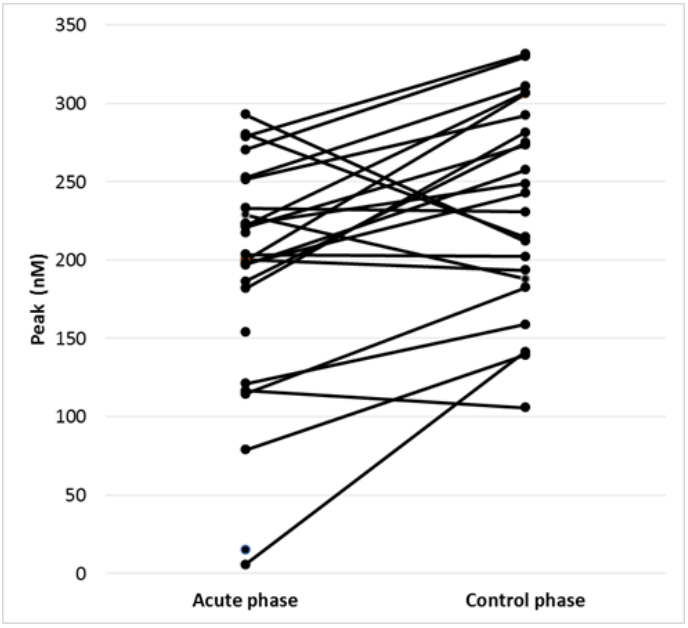
Abbreviations: ETP = endogenous thrombin potential

Figure 3. Endogenous thrombin potential (ETP) a), peak b), lag time c) and time to peak d) during acute phase (n=27) and control phase (n=23) of acute PUUV infection.

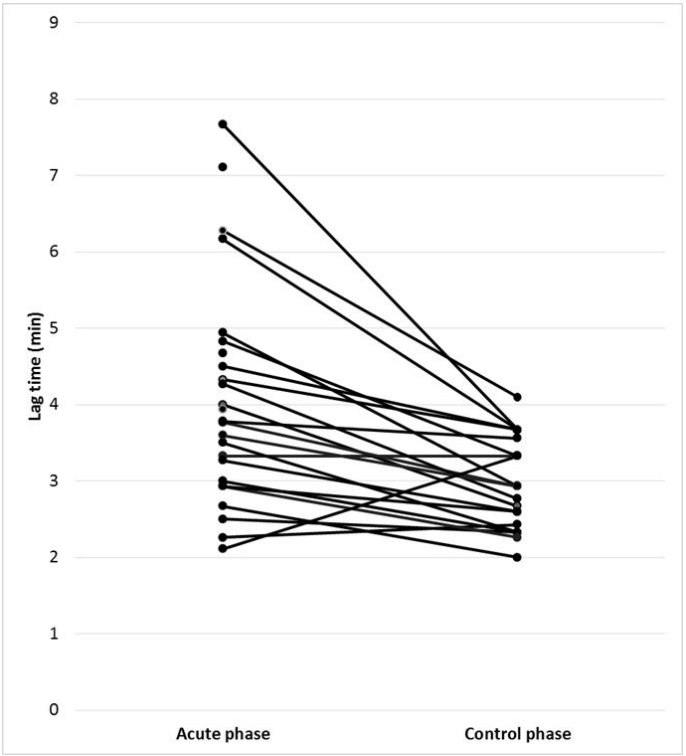
a)



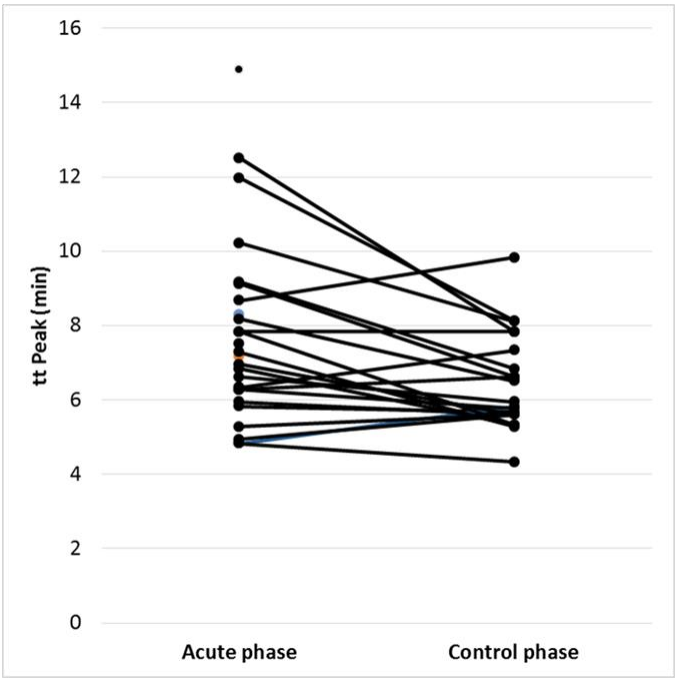
b)



c)


9
8
7
6
5
4
3
2
1
0
Lag time (min)
Acute phase
Control phase

d)


16
14
12
10
8
6
4
2
0
tt Peak (min)
Acute phase
Control phase

5.3.1 Associations of CAT® parametrs and variables depicting coagulation and disease severity

D-dimer was increased in the acute phase (median 2.8 mg/l, range 0.6-34 mg/l vs. median 0.4 mg/l, range 0.2-1.1 mg/l; p<0.001). D-dimer associated strongly with prothrombin fragments, F1+2 (r=0.843, p<0.001). F1+2 was elevated acutely (704 pmol/l, 284-1875 pmol/l vs. 263 pmol/l, 118-556 pmol/l; p<0.001). The fibrinogen level was also increased acutely compared to control (median 4.2, range 2.2-9.6 g/l and median 3.4, range 2.6-4.9 g/l; p=0.005). Prolonged LT and decreased ETP associated with increased fibrinogen level in the acute phase (r=0.511, p=0.006 and r=0.386, p=0.047, respectively).

ETP did not associate with D-dimer and F1+2 (r=-0.079, p=0.695 and r=-0.164, p=0.415, respectively). Furthermore, there were no associations found between peak and D-dimer and F1+2 (r=-0.030, p=0.882 and r=-0.162, p=0.418, respectively). ETP and peak thrombin concentration were associated with the platelet count measured in the CAT® study day sample (r=0.472, p=0.015 and r=0.554, p=0.003, respectively), and with the lowest platelet count recorded during acute phase (r=0.402, p=0.038 and r=0.462, p=0.015, respectively).

Clinical variables depicting the disease severity, i.e. maximum leukocyte count, maximum creatinine, CRP level and length of hospital stay did not associate with CAT® parameters (data not shown). Minor bleedings were reported in eight patients, but no associations could be detected with ETP and peak (data not shown).

5.4 Genetic polymorphisms of eNOS and iNOS (IV)

The genotype distributions and allele frequences for eNOS (rs1799983) and iNOS G2087A (rs2297518) are presented in Table 7. The genotyping was successfully performed in 167 patients for eNOS and in 166 for iNOS from 172 studied patients with PUUV infection. The clinical and laboratory variables of the study population are

shown in Table 1 in the original publication. Three patients (2%) were in clinical shock at the time of admission. Seven patients (4 %) were in need of hemodialysis treatment. AKI (plasma creatinine ≥135 μmol/l in women, and ≥150 μmol/l in men) was encountered in 96 (56 %) patients.

Table 7. The distributions of genotypes and allele frequencies in 167 patients for eNOS G894T(rs1799983) and in 166 patients for iNOS G2087A(rs2297518) out 172 patients with acute Puumala hantavirus infection.

	Genotype %			Allele %	
Polymorphism	homotsygote common	heterotsygote rare	homozygote	common	rare
eNOS (rs1799983)	59 (GG)	35 (GT)	6 (TT)	76 (G)	24 (T)
iNOS (rs2297518)	65 (GG)	33 (GA)	2 (AA)	81 (G)	19 (A)

eNOS= endothelial nitric oxide synthase, iNOS=inducible nitric oxide synthase.

5.4.1 Associations of eNOS G894T polymorphism with the clinical disease

The rare genotype of eNOS G894T (TT-homozygote, n=10) was found to associate with severe AKI in acute PUUV infection. Three out of the seven (43 %) patients that needed hemodialysis treatment were T-allele carriers, and one of them was TT-homozygote. Patients with TT-homozygous genotype (non-carriers of G-allele) suffered from more severe AKI compared to GT-heterozygotes and GG-homozygotes (median maximum creatinine 326, range 102-1041 vs. median 175, range 51-1499 μmol/l; p= 0.018, respectively, Figure 1A in the original publication). TT-homozygotes had the highest plasma creatinine value compared to GT-heterozygotes and GG-homozygotes (median 325 μmol/l, 196 and 166 μmol/l, respectively; p=0.061).

Furthermore, patients with TT-homozygous genotype of eNOS G894T polymorphism showed higher maximum blood hematocrit level than GT-

heterozygotes and GG-homozygotes genotypes (median 0.49, range 0.39-0.59 v. median 0.44, range 0.33-0.60; p=0.025, respectively, Figure 1B in original publication). The maximum leukocyte count was higher in the non-carriers of G-allele than G-allele carriers of eNOS G894T gene variant (median 12.9, range 8.1-26.8 vs. median 9.9, range 3.9-31.2 x 10^9/l; p= 0.047, Figure 1C in original publication). Also, the length of hospital duration was longer in TT-homozygous patients compared to the other genotypes (median 8 days, range 3-14 days vs. median 6 days, range 2-15 days; p= 0.032). ENOS G894T polymorphism did not associate with blood pressure level, change in weight, lowest platelet count, maximum CRP, maximum plasma IL-6 or the need of hemodialysis treatment (data not shown).

5.4.2 Associations of iNOS G2087A polymorphism with the clinical disease

Gene polymorphism of iNOS G2087A associated with hypotension among the carriers of rare A-allele (GA-heterozygotes and AA-homozygotes, n=59) had both lower minimum systolic and diastolic blood pressure level measured than the non-carriers (median 110, range 74-170 vs. 116, range 86-162 mmHg, respectively; p= 0.019, and median 68, range 40-90 vs. 72, range 48-100 mmHg, respectively; p= 0.003). The studied iNOS polymorphism did not associate with other clinical variables depicting clinical disease, change in weight, the lowest platelet count, maximum CRP, maximum plasma IL-6 or the need of hemodialysis treatment (data not shown).

6 DISCUSSION

6.1 Splenomegaly and association with clinical disease (I)

An enlarged spleen was detected in the MRI study in all studied patients during the acute phase of PUUV infection. The spleen length was noted to be reversed in the control phase MRI, which was carried out mean 196 days from the onset of symptoms. The median change in splenic length was 15 mm (range 7-22 mm) and the longest spleen observed was 154 mm. Thus, a moderate splenomegaly can be frequently observed during hantavirus infection by radiological imaging as an organ involvement. This finding is in line with the previous literature concerning other viral infections associated with splenomegaly. In Ebstein Barr virus infection splenomegaly can be detected by ultrasonography in almost all cases, but is variably noted in physical examination (Luzuriaga and Sullivan 2010).

Previous reports about spleen in hantavirus infections include a case of splenic rupture, a potentially life-threatening complication (Alexeyev et al. 1994). Also a few cases of hemophagocytic syndrome and hepatosplenomegaly in PUUV- and HTNV-caused HFRS have been reported (Baty et al. 1998, Lee et al. 2002, Park et al. 2011). In a German study with 64 PUUV-infected patients, in 24 patients (55.8 %) splenomegaly was present in ultrasonography (Sadeghi et al. 2011).

Thrombocytopenia due to enhanced splenic sequestration may occur in different conditions that cause splenomegaly. Almost 90% of all circulating platelets can be revesibly pooled in the enlarged spleen (Aster RH 1966), which causes decreased platelet count in circulation. Normally one-third of the platelets that are produced in the bone marrow are stored in the spleen. In this study the nadir platelet count measured during hospital stay, showed no association with the change of splenic

length. Thus, this data does not support the role of splenic sequestration in the pathogenesis of decreased platelet count in PUUV-HFRS.

Splenomegaly was associated negatively with the highest leukocyte count. This may be suggestive of increased pooling of leukocytes in the enlarged spleen. Furthermore, the maximum CRP level was moderately associated with splenomegaly. Other clinical variables like the highest creatinine value, length of hospital stay and the mininum or maximum hematocrit values were not associated with enlarged spleen. Furher, no correlation with the change in body weight was found.

Splenomegaly as an organ involvement is probably linked to the immunopathogenesis of hantavirus infection. Enlarged spleen could possibly be a consequence of the proliferation of mononuclear cells as an immune response directed by cytotoxic T-lymphocytes, regulatory T-cells and proinflammatory cytokines. On the other hand, the red pulp of the spleen tissue functions as a blood filter of micro-organisms, antigens and old blood cells. It is likely that the enlarged spleen is due to enhanced function as a response to the viral infection. PUUV RNA has been detected in the splenic endothelial capillaries of macaque monkeys (Sironen et al. 2008). According to a recent study, in autopsies of four fatal PUUV-cases high amounts of PUUV nucleocapsid protein N were detected, mostly in the macrophages in the spleen sinusoides along with hemorrhages and congestion (Sironen et al. 2017). Furthermore, previously described two severely ill PUUV-patients with extensive capillary leakage treated with icatibant have been susceptible to spleen deficiency (Antonen et al. 2013, Laine et al. 2015b).

Abdominal pain is a common symptom during hantaviral disease. Physical examination to detect possible splenomegaly can be unreliable, thus in severe cases evaluation of spleen size by abdominal imaging can be of clinical importance to get insight into a patient`s spleen.

6.2 Association of coagulation and fibrinolysis variables with complement activation (II)

Study II showed numerous associations between disease predictive biomarkers and hemostatic variables. Especially PTX3, an acute phase reactant rapidly produced at the site of inflammation, associated strongly with different variables reflecting thrombin formation and activation of both coagulation and endothelium. Lowered level of plasma AT associated with high PTX3, low complement component C3, high IL-6 and cf-DNA.

Increased PTX3 levels correlated strongly with diminished levels of natural anticoagulants, like AT, PS free antigen and PC. Also, high PTX3 values associated with prothrombin fragments F1+2, suggestive of thrombin generation and lowered endothelial marker ADAMTS13. Furthermore, PTX3 associated with the consumption of platelet ligand fibrinogen. PTX3 is known to activate the classical complement cascade by binding the complement component C1q (Botazzi et al. 2010). It can also interact with factor H, the alternative complement pathway regulator (Deban et al. 2008). It has also previously been shown that PTX3 associates with the terminal increased complement component complex SC5b-9 and inversely with the C3 levels (Outinen et al. 2011). In addition, PTX3 interacts with P-selectin which is released from Weibel-Palade bodies, the storage granules of endothelial cells, and from platelet α-granules (Deban et al. 2010). Elevated levels of PTX3 have previously been shown to indicate more severe clinical disease and thrombocytopenia in acute PUUV infection (Outinen et al. 2011). Taken together, study II findings further emphasize the role of PTX3 in the crosstalk between coagulation, complement activation and inflammation in acute PUUV-HFRS.

In study II, decreased plasma levels of AT associated with high PTX3, Cf-DNA, IL-6 and low plasma C3 are predictive markers for disease severity in PUUV-HFRS. These four markers have previously been shown to associate with thrombocytopenia, high leukocyte count and longer hospital stay (Outinen et al. 2010, Outinen et al. 2011, Sane et al. 2012, Outinen et al. 2012). AT functions as a serine protease inhibitor that

inhibits coagulation enzymes, especially thrombin and activated coagulation factor X. Furthermore, it inhibits both classical pathway of complement and lectin pathway complement enzymes. AT also has anti-inflammatory properties independent of its effect on coagulation. AT induces endothelial cell release of prostacyclin, and thus suppresses platelet activation and aggregation, inhibits the adhesion of neutrophils to the endothelium and decreases production of cytokines IL-6 and TNF from endothelial cells. AT inhibits leukocyte activation also by downregulating P-selectin activity (Levy et al. 2016). AT levels can be diminished due to impaired liver synthesis, degradation by elastase from activated neutrophils, removal from circulation because of capillary leakage, and consumption of thrombin generation (Levy et al. 2016). The two latter ones are encountered during acute hantavirus infection (Laine et al. 2010, Mustonen et al. 2013).

Study II also showed that elevated plasma cf-DNA, considered to be originated from apoptotic cells or neutrophil extracellular traps (NETs), correlated with prothrombin fragments, F1+2 and APTT the screening coagulation test. Also, associations with complement activation SC5b-9 and C3 were found. The capacity of hantaviruses to induce NET formation through ß2 signaling is demonstrated to contribute to the disease immunopathology (Raftery et al. 2014). In addition, increased plasma cf-DNA levels associate with PTX3 during acute PUUV-HFRS, probably explained by opsonisation and clearance of apoptotic and necrotic cells (Outinen et al. 2012). NETosis is considered important in host defense through innate immunity, but also through procoagulant mechanisms. NETs support histones and neutrophil DNA fragments to induce coagulation activation during sepsis and inflammation (Delabranche et al. 2017). Histones recruit and activate platelets, and negatively charged DNA provides an activated surface for coagulation factors assembly. Furthermore, neutrophil elastase, an enzyme released from NETs, can inhibit tissue factor pathway inhibitor (Massberg et al. 2010) and thrombomodulin (TM), thus impairing protein C pathway (Ammolo et al. 2011).

In study II low C3 levels indicating the alternative pathway activation of complement system associated with the loss of natural anticoagulants, PC and PS free

antigen as well with APTT. The activation of the complement system through both classical and alternative pathways is involved in the PUUV-HFRS pathogenesis and associates with the disease severity (Paakkala et al. 2000, Sane et al. 2012). Study II findings are in concordance with an earlier study reporting associations between diminished C3 levels and coagulation factors during Hantaan virus-induced HFRS (Lee, 1987).

Finally, study II showed no associations between PTX3, IL-6, cf-DNA, S-C5b-9 and C3, all markers describing severe hantaviral disease, and increased level of plasma D-dimer. Enhanced fibrinolysis indicated by high plasma D-dimer level has been reported in PUUV-HFRS (Laine et al. 2010). However, it does not seem to predict the severity of disease.

The small sample size of study population is one limitation of studies I-II. Although various significant associations between markers of coagulation, fibrinolysis and complement system could be established, findings of study II do not prove causality between them. There were also some overlapping with the timing of blood samples taken, because the patients sought to the hospital care at various time points from the onset of disease.

6.3 Thrombin generation by CAT® (III)

In study III, the thrombin generation (TG) in the platelet poor plasma (PPP) of 27 patients with PUUV-HFRS was analysed by Calibrated automated thrombogram (CAT®). The possible associations of CAT® with traditional coagulation tests applied in clinical practice and variables depicting disease severity were evaluated. The study aim was to describe how plasma TG potential is rendered in the acute and recovery phase of infection. CAT® assay is known to measure *in vitro* TG of plasma, thus indicating the overall haemostatic capacity of plasma (Hemker et al. 2002). Both hemorrhagic and thrombotic events are known to associate with clinical hantavirus

disease, but the underlying mechanisms of alterations in the coagulation system need to be better clarified.

Study III showed that both endogenous thrombin potential (ETP) and peak thrombin concentration were decreased during acute phase of PUUV-HFRS compared with the recovery phase. PUUV-infected patients showed a prolonged lag time (LT) and time to reach the peak thrombin concentration after initiation of TG by a triggering reactant including 5 pM tissue factor and 4 μM phospholipids without corn-trypsin-inhibitor. According to an earlier study, slightly prolonged PT, prolonged APTT, and shortened thrombin time (TT) have been reported (Laine et al. 2010). *In vivo* coagulation tests APTT and PT measure the time to the beginning of clot formation, and thus they do not fully reflect the hemostatic balance in acutely ill patients. These plasma clotting tests are considered to correlate with the LT phase of TG in CAT®. Thus, the finding of a prolonged LT, indicating a delayed onset of TG, is in line with previous findings about coagulation tests APTT and PT. However, since the data involving *in vitro* coagulation tests was not available, a direct comparison with CAT® variables was not possible.

Previous studies suggest increased thrombin generation indicated by an increased amount of circulating prothrombin fragments 1+2 (F 1+2) and D-dimer, the fibrin degradation product and lowered levels of antithrombin (AT), protein C (PC) and protein S (PS) (Laine et al. 2010, Sundberg et al. 2011). In line with the earlier studies, study III also showed increased levels of F1+2 and D-dimer in the acute phase. *In vivo* TG parameters, F1+2 and D-dimer, depend on the amount of TF and TM, that is expressed on the endothelial cells and serves as cofactor for thrombin reducing blood coagulation (Dieri et al. 2012). In addition, TG is ongoing *in vivo* in microparticles released during acute PUUV infection (Owens et al. 2011, Laine et al. 2016). Previous studies also indicate increased TF amounts in endothelial cells in the acute phase of PUUV-HFRS (Goeijenbier et al. 2015). Study III showed an association with low platelet count and low ETP and low peak, which may suggest thrombin activation and consumption of platelets, since thrombopoiesis is demonstrated to be active in acute PUUV-HFRS (Connolly-Andersen et al. 2015, Laine et al. 2016). Increased TF

expression on the endothelial cells and microparticle release might result in consumption of platelets and clotting factors resulting in lower ETP *in vitro*. This is supported by the increased amounts of D-dimer and F1+2, *in vivo* markers of coagulation activation. Natural anticoagulants, PS, PS free antigen and AT are shown to be decreased (Laine et al. 2010, Laine et al. 2014).

The discrepancy of various study results can be better explained by different methods to measure thrombin formation. CAT® assay measures *in vitro* TG capacity of coagulation pathways after a predetermined activation trigger (Baglin 2005). Thus, it measures the haemostatic balance of plasma clotting factors and inhibitors independently of the procoagulant and inhibitory drivers released from the endothelium (Tripodi 2016). On the other hand, measurements to evaluate *in vivo* TG, for example fibrin degradation products, such as D-dimer, prothrombin fragment F1+2 or enzyme-inhibitor complexes, such as thrombin-antithrombin, are also influenced by fibrinolytic activity and clearance times. However, clinical utility to assess the likelihood of bleeding and thrombosis tendency can be obtained from combined measurements. The difference between *in vivo* and *in vitro* TG can be observed in consumption coagulopathy, where the parametrs of ongoing coagulation are elevated, although the plasma capacity of TG is diminished (Seo et al. 2009).

Fibrinolysis is enhanced, according to elevated levels of D-dimer and endothelial cell tPA in acute PUUV-HFRS (Laine et al. 2010, Laine et al. 2014, Goeijenbier et al. 2015, Strandin et al. 2016). PAI-1 level is not altered in the acute phase (Strandin et al. 2016). The findings of diminished TG together with previous knowledge of enhanced fibrinolysis in PUUV-HFRS have similarities with studies of another hemorrhagic fever, dengue virus infection (Sosothikul et al. 2007, Van de Weg et al. 2014). In dengue fever the bleeding complications have been demonstrated to associate with decreased TG along with thrombocytopenia and enhanced fibrinolysis (Orsi et al. 2013). In study III, no statistically significant correlations between decreased ETP and bleeding symptoms were found. Although, minor bleedings were reported in one third of the study subjects.

One limitation of study III is the quite small sample size of patients. Also, the traditional *in vivo* coagulation tests were not available to make a comparison with CAT® findings. Two patients were on an aspirin drug, a platelet antagonist inhibiting platelet aggregation and TG formation, but CAT® analysis were made in PPP. However, it is unlikely that minor residual platelets might affect these study results.

To conclude, decreased ETP together with low peak and prolonged lag time indicate decreased plasma potential for thrombin generation *in vitro*. Together with thrombocytopenia, enhanced fibrinolysis and signs of increased TG *in vivo* this data suggests a mild to moderate consumption coagulopathy during acute PUUV infection. These CAT® results concerning plasma hypocoagulobility support previous findings of impaired hemostasis during acute PUUV-HFRS.

6.4 Polymorphisms of eNOS, iNOS and clinical disease (IV)

Increased serum nitric oxide (NO) levels induced by IFN-γ, TNF-α and other cytokines have been implicated in the pathogenesis of hantaviral disease (Linderholm et al. 1996, Davis et al. 2002). NO overproduction during PUUV-HFRS seems to correlate with increased serum creatinine value and hypotension as well decreased platelet count (Groeneveld et al. 1995). Endothelial nitric oxide synthase (eNOS) expressed in the endothelial cells direct NO production, which suppresses platelet activation and aggregation and potentially vasodilates vessels to increase circulation. Inducible nitric oxide synthase (iNOS), expressed in macrophages, produces high amounts of NO upon stimulation of various cytokines as a host immune response (Green et al. 1994). Previous knowledge about the role of genetic polymorphisms of nitric oxide synthases (NOS) in hantaviral infection are lacking.

Study IV showed that eNOS gene polymorphism G894T (Glu298Asp) was associated with clinically severe hantaviral disease. The studied eNOS polymorphism G894T (Glu298Asp) results in a substitution of glutamate for aspartate at position 298 in eNOS exon 7, which renders this molecular variant susceptible to proteolytic

cleavage, decreased enzymatic activity and decreased basal NO synthesis in the blood vessels (Veldman et al. 2002, Erbs et al. 2006). NO, produced by renal mesangial and tubular cells, is a significant regulator and a protector of renal blood flow, glomerular filtration rate and tubular function (Modlinger et al. 2004).

Study IV showed that patients with the rare TT-homozygous genotype (6%) had more severe AKI as evaluated by the highest maximum creatinine level measured, compared with the GT-heterozygous and GG-homozygous genotypes of patients. In addition, the need of hemodialysis treatment during hospital stay was more frequent among T-allele carriers. PUUV-infected patients with the common GG-genotype showed a milder clinical disease than other genotypes. These findings are in concordance with the previous ones that T-allele of eNOS G894T polymorphism associates with impaired renal hemodynamic function and end-stage renal disease susceptibility, whereas GG-genotype is implicated to play a protective role (Cherney et al. 2009. Yun et al. 2014). Also study IV showed that TT-homozygotes had higher maximum leukocyte count, hematocrit values measured and longer hospital stay than GT-heterozygotes or GG-homozygotes. The G894T polymorphism of eNOS gene was not associated with the depth of thrombocytopenia, which is in line with earlier studies that in Finnish PUUV-infected patients the severity of AKI does not seem to associate with thrombocytopenia (Libraty et al. 2012). However, in German studies severe thrombocytopenia has implicated a severe AKI during PUUV-HFRS (Rasche et al. 2004, Latus et al. 2015). These divergent results may be explained by genetic variety in study populations.

Study IV also evaluated the possible associations of iNOS gene variant G2087A with the disease severity of PUUV-HFRS. This gene polymorphism that leads to a substitution from serine to leucine in the coding region of exon 16 in NOS2, was associated with hypotension during acute phase. Especially the rare A-allele carriers suffered from low blood pressure measured during hospital care. This finding is in line with the studies suggesting that A-allele carriage has been associated with excessive NO production and inflammatory response and with susceptibility to septic shock via enhanced iNOS activity (Wang et al. 2006, Wang et al. 2013). INOS G2087A

polymorphism was not associated with change of weight, lowest platelet count, maximum CRP level, maximum plasma IL-6 and maximum creatinine level.

To conclude, eNOS G894T polymorphism was shown to associate with the disease severity of PUUV-HFRS. TT-homozygous genotype may predispose PUUV-infected patients to an impaired renal function possibly through reduced constitutive NO bioavailability. Thus, this eNOS gene variant, which has been linked to various vascular diseases, may be involved in the pathogenesis of endothelial and kidney dysfunction. In addition, these study findings support the previous studies indicative of host genetic factors contributing to the clinical outcome of PUUV-HFRS.

6.5 Future considerations

Knowledge concerning coagulation activation and thrombocytopenia during hantavirus infection has increased in the recent years. The complement system have been shown to be activated via both alternative pathway and classical route (Paakkala et al. 2000, Sane et al. 2012). Acutely elevated complement complex SC5b-9 is capable of enhancing capillary permeability via ligating ß3-integrin, releasing BK and platelet-activating factor (Tsukada et al. 1995, Bossi et al. 2004). Previous case-reports about treating severely ill PUUV-infected patients with icatibant suggest that further studies with BK receptor antagonist in the treatment of severe hantavirus disease are needed (Antonen et al. 2013, Laine et al. 2015b).

Hantaviruses are demonstrated to be able to induce NET formation through ß2 signalling (Raftery et al. 2014). Histones are major constituents of NETs that can promote platelet and endothelial activation (Fuchs et al. 2011). Histones also hamper AT mediated neutralization of thrombin and enhance autoactivation of prothrombin to thrombin (Barranco-Medina et al. 2013). Thus, it woud be interesting to evaluate the role of histones and NETs underlying thrombocytopenia and coagulation activation in hantavirus disease.

TG was measured in PPP by CAT®, thus it was done in the absence of platelets, erythrocytes and other cells that can contribute to TG. In comparison, it would be enlightning to also assess thrombin formation in platelet rich plasma TG analysis with a larger study population. This might further clarify the role of TG in the context of bleeding and thrombosis during hantavirus infection.

As previously discussed, polymorphisms of enzymes catalysing NO production in the endothelium and various cells, were found to be associated with clinically severe PUUV infection. To further investigate the role of these polymorphisms in the pathogenesis of endothelial dysfunction, variables indicating endothelial and coagulation activation could be measured and compared in patients with and without these certain gene variants.

7 SUMMARY AND CONCLUSIONS

The main findings of the studies in this thesis can be summarized as follows:

I Enlarged spleen is a frequently detected lymphoid organ involvement in abdominal MRI in PUUV-infected patients. Splenomegaly is associated inversely with high leukocyte count and positively with CRP level, but not with low platelet count.

II Disease predictive markers PTX-3, cf-DNA, complement proteins C3 and SC5b-9 and IL-6 have several associations with markers reflecting endothelial activation, coagulation and fibrinolysis during acute PUUV infection. High plasma PTX-3 level associate with thrombin formation, consumption of platelet ligand fibrinogen and natural anticoagulants, AT, PC and protein S free antigen and reduced endothelial marker ADAMTS13.

III Decreased ETP together with low peak thrombin concentration and prolonged LT were found during acute phase of PUUV infection by CAT® suggesting impaired plasma capacity for TG *in vitro*. Thrombocytopenia associated with low ETP and peak, but no associations with clinical variables describing disease severity and FIDD and F1+2 could be detected.

IV Polymorphism of eNOS G894T is associated with PUUV-induced AKI and clinically severe disease. Polymorphism of iNOS G2087A is associated with hypotension during PUUV infection.

In conclusion, a moderately enlarged spleen was noted in all patients in MRI examinations, further emphasizing the role of the immune system in the disease pathogenesis. However, enhanced splenic sequestration of platelets as an underlying cause of thrombocytopenia does not seem to play as significant a role. Enlarged spleen

was associated with inflammatory laboratory variables, such as the highest CRP level and inversely with the highest blood leukocyte count, but not with other clinical variables reflecting severe disease outcome or AKI.

Variables indicating coagulation, complement and endothelial activation, were shown to follow the predictive markers of disease severity, mainly PTX-3, during acute PUUV infection. In addition, depleted levels of plasma AT correlated with high plasma levels of PTX3, cf-DNA, IL-6 and low plasma C3. These study results highlight the multiple connections between the host`s inflammatory response and coagulation pathways in PUUV-induced HFRS and are concordant with the previous findings of these biomarkers also being predictive for thrombocytopenia.

In CAT® analysis, thrombin generation capacity of plasma was found to be decreased and prolonged during acute phase compared to the recovery. Low platelet count and fibrinogen level followed decreased ETP and peak thrombin concentration. Taken together with thrombocytopenia, loss of physiological anticoagulants and enhanced fibrinolysis, the finding of a diminished *in vitro* TG potential of plasma further shifts the overall hemostatic balance toward the hypocoagulable state.

Polymorphisms involved in NO synthesis were found to associate with the clinical outcome of PUUV infection. TT-homozygous for eNOS G894T gene variant were more susceptible to severe AKI, longer hospital stay, higher blood leukocytosis and hemoconcentration compared with other genotypes. This gene variant may play some role in the endothelial dysfunction via decreased NO production in the endothelium during acute PUUV infection. Gene polymorphism of iNOS G2087A was associated with hypotension, especially among the A-allele carriers.

8 ACKNOWLEDGEMENTS

This study was carried out at the Department of Internal Medicine, Tampere University Hospital, and at Faculty of Medicine and Life Sciences, University of Tampere.

I want to express my deep gratitude to my supervisor Professor emeritus Jukka Mustonen, M.D., for giving me the opportunity to conduct this dissertation in his guidance. Jukka is a truly devoted scientist with a vast knowledge of hantavirus diseases, and his enthusiasm and encouragement have been invaluable to this work to be completed. Jukka always had time for my questions and this project. His positive attitude and way of thinking have created a good spirit among our research group. I have been privileged to be introduced to scientific research by him.

I am deeply grateful to my other supervisor, Docent Outi Laine, M.D., for initially suggesting this thesis for me. Her continuous guidance and encouragement towards scientific research, and all her support have been essential to me throughout the study years. I appreciate the expertise in the field of hemostasis and persistent effort Outi has provided into this project. I am also thankful for all the practical advice, good company and many laughs during this journey.

My warmest thanks belong to a member of my thesis committee, Docent Satu Mäkelä, M.D., for sharing her wide experience and knowledge of scientific research, hantavirus diseases and study protocols. Her supportive attitude and clever thinking have been important to this study from the beginning. Satu`s kind help in teaching me the basics in statistics and scientific writing have been invaluable.

I sincerely thank Docent Lotta Joutsi-Korhonen, M.D., a member of my thesis committee, for bringing her expertise in clinical laboratory and field of coagulation into this dissertation. Her bright and valuable comments have certainly improved the quality of this work. It has been a great pleasure to get to know her.

I am very thankful to Professor Ilkka Pörsti, M.D., for valuable comments, language edition and assistance with the figures of the original articles.

I am sincerely grateful to my co-authors for collaboration, Tuula Outinen, M.D., Ph.D., Professor Antti Paakkala, M.D., Professor Antti Vaheri, M.D., Professor Pekka Karhunen, M.D., Tanja Pessi, Ph.D., Sari Tuomisto, Ph.D., Professor Mikko Hurme, M.D., and Juulia Jylhävä, Ph.D. My warmest thanks go to Heini Huhtala, M.Sc., for conducting and helping me with the statistical analyses and figures for my articles.

I am thankful to the heads of the Department of Internal Medicine, Docent Heikki Oksa, M.D., Docent Kari Pietilä, M.D., Docent Jaakko Antonen, M.D. and Professor Katri Kaukinen, M.D., for giving me the opportunity and providing the facitilites to carry out this project in the clinic. I appreciate their supportive attitude toward research work.

I am truly grateful to Docent Marjatta Sinisalo, M.D., for her supportive attitude towards research and flexibility concerning my study leaves at the Hematology Unit.

I am thankful to the study nurses, Katriina Yli-Nikkilä and Eini Eskola for their excellent and friendly assistance with the study protocols and patients. I warmly thank Sirpa Järvensivu for the revision of English in this book.

I am grateful to the reviewers of this dissertation, Docent Sakari Kakko, M.D., and Docent Risto Ikäheimo, M.D., for investing their time and knowledge in reviewing and improving the quality of this thesis.

I wish to thank all my colleagues and friends at the Department of Internal Medicine for the encouragement and creating a friendly environment to work at. Above all, I thank my dear colleagues and friends at the Hematology Unit for making it such a nice workplace with their joyful company.

I sincerely thank my parents for all their support and help throughout the years. My twin sister, Päivi Koskela-Karjalainen, M.D., is thanked for her friendship and support as well as continuous encouragement towards this project.

Finally, I wish to express my deepest gratitude to my family. Mikko, without your love, positive attitude and endless support this thesis would never have been possible. I am very proud of our precious daughters Aada, Veera and Nella, who remind me every

day what is truly important in life. You bring such happiness and activity into my everyday living.

This study was financially supported by Competetive Research Funding of the Pirkanmaa Hospital District, the Blood Research Foundation, the Finnish Association of Hematology, the Finnish Kidney Foundation, Maud Kuistila Foundation and Orion Research Foundation.

Kangasala, June 2018
Sirpa Koskela

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Spleen enlargement is a common finding in acute Puumala hantavirus infection and it does not associate with thrombocytopenia

Running headline: Spleen and thrombocytopenia in Puumala hantavirus infection

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Keywords: Puumala hantavirus; Thrombocytopenia; Spleen; Platelet; MRI

Declaration of interest:

Sources of support: the Competitive State Research Financing of the Expert Responsibility Area of Tampere University Hospital, the Blood Disease Research Foundation, the Finnish Kidney Foundation and Sigrid Jusélius Foundation

Conflicts of interest: there are no conflicts of interest.

Abstract

The pathogenesis of thrombocytopenia in Puumala hantavirus (PUUV) infection is probably multifactorial. We aimed to evaluate the possible spleen enlargement during acute PUUV infection, and to determine its association with thrombocytopenia and disease severity. Spleen magnetic resonance imaging (MRI) was performed in 20 patients with acute PUUV infection. MRI was repeated 5-8 months later. The change in spleen length was compared with markers describing the severity of the disease. In all patients, the spleen length was increased in the acute phase compared with the control phase (median 129 mm vs. 111 mm, $p<0.001$). The change correlated with maximum C-reactive protein value ($r=0.513$, $p=0.021$) and inversely with maximum leukocyte count ($r=-0.471$, $p=0.036$), but not with maximum serum creatinine level or minimum platelet count. Enlarged spleen, evaluated by MRI, was shown to be a common finding during acute PUUV infection. However, it does not associate with thrombocytopenia and acute kidney injury.

Introduction

Puumala hantavirus (PUUV) infection, also known as nephropathia epidemica (NE), is the most common cause of hemorrhagic fever with renal syndrome (HFRS) in Europe. Some 10 000 cases are reported annually in Europe and the highest numbers come from Finland [1]. Humans catch the disease from the aerosolized excreta of an infected rodent (Myodes glareolus*)*. The clinical course of PUUV infection varies from mostly mild to the rare fatal outcome. The symptoms begin with high fever, headache, backache, abdominal pains, visual disturbances, nausea and vomiting. The renal involvement is characterized by proteinuria, hematuria, increased serum creatinine levels and oliguria, followed by polyuria [1]. Although thrombocytopenia is common bleeding symptoms usually remain mild.

HFRS is characterized by altered vascular permeability together with thrombocytopenia [2, 3]. Possible pathophysiological mechanisms of increased permeability include activation of proinflammatory cytokines [4, 5] as well as cytotoxic CD8+ T lymphocytes [6] and complement system [7, 8]. As for the decreased platelet count the scarce evidence does not imply impaired platelet production [9]. Recent reports on PUUV infection indicate increased thrombin formation and fibrinolysis and alterations in platelet ligands and acute phase reactants related to endothelial functions [3]. Also elevated pentraxin-3 levels and plasma cell free DNA levels have been found to associate with thrombocytopenia and disease severity in PUUV induced HFRS [3].

Enhanced clearance or sequestration of platelets by the reticuloendothelial system (RES), as seen in disorders with splenomegaly, has not been studied in hantaviral infections. It is known that spleen may be temporarily enlarged in acute viral and bacterial infections. There are few case reports of splenomegaly and hemophagocytic syndrome along with hepatosplenomegaly during HFRS caused

by PUUV [10-12] and Hantaan virus [13, 14]. A case of PUUV- HFRS complicated by a haemorrhage of spleen has also been reported [15].

In this study we aimed to evaluate the possible spleen enlargement measured by magnetic resonance imaging (MRI) during acute PUUV infection. Further, we sought to determine the possible associations between the change in spleen length and platelet count as well as other clinical markers reflecting the clinical course of the disease.

Material and methods

The patients studied here also made up the population of a previous study determining renal MRI findings during acute PUUV infection [16]. Twenty consecutive patients (17 males, 3 females) hospitalized due to acute serologically confirmed PUUV infection in Tampere University Hospital, Finland, were included. Their median age was 36 years (ranging from 17 to 72 years). Seven (35 %) of the patients had one or more of the following chronic diseases: Crohn`s disease, essential hypertension, coronary artery disease, hypercholesterolemia, type II diabetes, depression and epilepsy. None of the patients had chronic kidney disease, signs of any liver disease or hematologic malignancy.

Two MRI examinations were performed for each patient. The first examination was carried out on admission to the hospital, 4–12 (mean 7) days from the onset of fever, in the acute phase of PUUV infection, and the second one 151–267 (mean 196) days from the onset of fever, in the control phase. MRI was performed using a scanner Signa Horizon Echospeed LX (General Electric, Milwaukee, WI) operating at 1.5 T, using torso phase array coil.

The maximum spleen length was calculated by the same radiologist (A.P.) on coronal T2 fast spin echo fat suppression sequence with advantage Windows 4.3 workstation (General Electric Medical System, Paris). Regarding both acute-phase and control-phase imaging, the calculations were made twice, at two weeks intervals. The mean of these two measurements was defined as an acute-phase or control-phase spleen length. Radiologist was unaware of the patients` clinical data and the measurement of the first time. The patients were used as their own controls by comparing the mean of spleen length in the acute phase to the mean length in the control phase. The parenchymal length of both kidneys determined previously [16] were also included in this study. As for the laboratory variables, blood count, serum creatinine and C-reactive protein (CRP) values were measured by standard methods mostly daily according to clinical needs.

The study was carried out in Tampere University Hospital, Finland. All patients gave written informed consent and the study was approved by the Ethics Committee of Tampere University Hospital.

Statistical analyses

The highest and lowest values of continuous variables measured in the acute phase were designated as maximum or minimum values. In order to describe the data, medians and ranges were given for continuous variables and numbers for categorical variables. The Wilcoxon`s test was used to compare the mean spleen length between the acute and control phases. Correlations were calculated by the Spearman`s rank correlation test. All p-values were two-tailed, and the statistical significance was considered at 0.05. Statistical analyses were performed using SPSS software version 20.

Results

Clinical and laboratory findings

The clinical and laboratory findings of the 20 patients are shown in Table 1. One patient (5%) needed hemodialysis treatment during hospital care. Fifteen patients had chest X-ray taken upon hospitalization, three of them (20%) had mild pathologic findings. Eighteen (90%) of the 20 patients had a minimum platelet count lower than 150 x 10^9/l and in five patients (25%) it was 50 x 10^9/l or lower. Twelve (60%) patients had a maximum leukocyte count higher than 10 x 10^9/l, ten (50%) had a plasma CRP value higher than 100 mg/l and 19 (95 %) patients had a maximum serum creatinine concentration over 100 µmol/l. All patients recovered.

MRI findings

In all 20 patients the spleen length was increased in the acute phase MRI study compared with the control phase MRI (median 129 mm, range 104-154 mm vs. median 111 mm, range 92-140 mm, $p<0.001$; Figure 1). The median change in the spleen length was 15 mm (ranging from 7 to 22 mm).

There was an association between the maximum spleen length and the renal length (the mean length of right and left kidney) in the acute phase MRI study ($r=0.448$, $p=0.048$). However, no association was found between the change in the spleen length and the change in the renal length.

Associations of MRI findings with the severity of the disease

The change in the spleen length did not associate with the minimum platelet count ($r=0.171$, $p=0.472$). Instead, the change associated moderately with the maximum CRP value ($r=0.513$, $p=0.021$). Interestingly, a moderate inverse correlation prevailed between the change in the spleen

length and the maximum leukocyte count (r=-0.471, p=0.036). The change in the spleen length did not associate with the maximum serum creatinine concentration, the length of hospital stay and the change in body weight (reflecting fluid retention during oliguric phase) or the minimum or the maximum hematocrit (data not shown).

Discussion

In this study we assessed the spleen MRI findings during acute PUUV infection in 20 patients. We related the findings to the clinical course of the disease and the renal MRI findings determined previously [16]. Measurable changes in the spleen length occurred in all patients, and the median change between the acute and control phase MRI examinations was significant. Thus, spleen enlargement was noted in all 20 patients. The low platelet count, characteristic of acute PUUV infection, did not associate with the spleen enlargement. To our knowledge, this is the first study to assess the change in spleen size evaluated by MRI together with the clinical outcome of PUUV infection.

Spleen, considered the largest lymphoid organ, plays an essential role in the regulation of the immune system. The spleen acts as a filter by removing abnormal blood cells and foreign particles by its large population of mononuclear cells. Erytrophagocytosis, reflecting the removal of senescent red cells, increases as a result of viral infection [17]. The average size of a spleen is 10.9 ±1.4 cm in craniocaudal length [18] but it varies individually and correlates with the height of a person [19]. In this study we assumed that the spleen length in the control phase MRI (performed mean 196 days from the onset of symptoms) represented the normal spleen length of a subject. In our study the median spleen length in the control was 11.1 cm, which is in line with the estimates reported in the literature.

The spleen is usually considered enlarged when its length exceeds 11 cm [18]. The most common viral infections causing spleen enlargement are human immunodeficiency virus (HIV) infection, acute mononucleosis, dengue fever, rubella, cytomegalo virus and herpes simplex virus infections [17]. In infectious mononucleosis caused by Epstein-Barr virus (EBV) splenomegaly can be clinically noted in half of the patients, and almost in all patients if evaluated by ultrasound [20]. Our data demonstrate that a spleen enlargement is a frequent finding also during acute PUUV infection.

About one third of platelets are normally sequestered in the spleen [17]. Splenomegaly may contribute to thrombocytopenia by inducing a reversible pooling of up to 90 % of total body platelets despite normal thrombocytopoiesis, total body platelet mass and platelet life-span [21]. Usually the mechanism underlying the low platelet count in viral infections is decreased platelet production or increased platelet antibody formation, but also platelet sequestration due to splenomegaly may contribute [22]. In the present study no association was found between the nadir platelet count and the spleen enlargement. This implies that the enhanced clearance or pooling of platelets in the enlarged spleen might not be involved in the pathogenesis of thrombocytopenia in PUUV infection.

The high leukocyte count was found to associate inversely with the change in spleen length. This may suggest increased pooling of leukocytes in the enlarged spleen. Also another inflammation marker, the maximum CRP value, correlated with the spleen enlargement. These findings may indicate that splenomegaly in acute PUUV infection might be due to an immunologic response to the viral antigenic stimulation. In animal studies, PUUV RNA has been observed in the spleen, mostly in the endothelial capillaries [23].

In our previous study determining the renal MRI findings, enlargement of kidneys were found to associate weakly with fluid volume overload, thrombocytopenia and acute kidney injury (AKI) [16]. In this study we found no such associations indicating that the spleen enlargement does not relate to fluid accumulation or severity of AKI. No association was found between the change in the renal size and the change in the spleen size, either. All this may suggest a different pathogenic mechanism in the increase of various organs sizes.

We conclude that the enlargement of spleen is a common finding during acute PUUV infection. The enlargement has no association with the low platelet count or markers reflecting the severity of AKI. Thus, enhanced sequestration of platelets by the spleen does not seem to play any specific role in the pathogenesis of thrombocytopenia encountered in hantaviral diseases. The spleen enlargement associates inversely with the blood leukocyte count, which might reflect the pooling of leukocytes in the enlarged spleen.

Acknowledgements

The skillful technical assistance of Ms Katriina Ylinikkilä is greatly appreciated.

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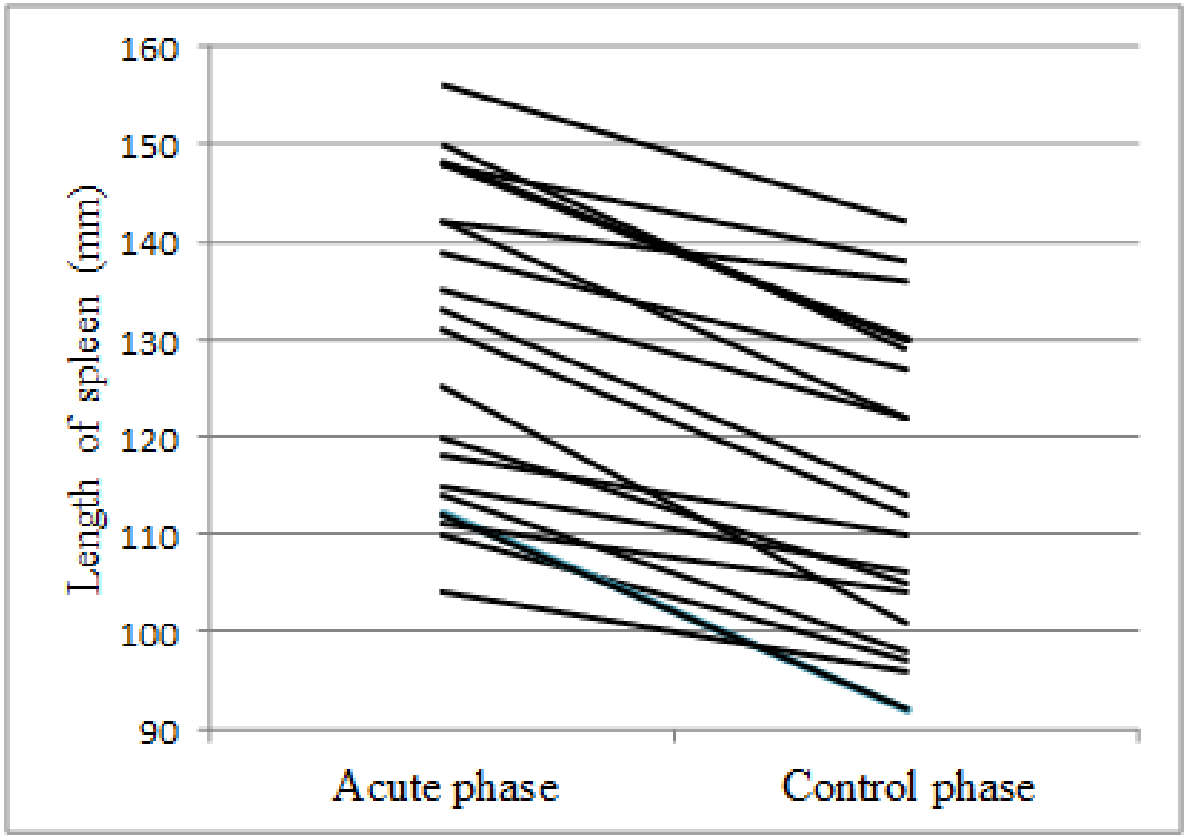
Legend for Figure 1.

The length of spleen in 20 patients with acute PUUV infection studied by MRI in the acute and in the control phase.

Table I. The clinical and laboratory findings in 20 patients with acute Puumala hantavirus infection.

Clinical and laboratory findings	**Median**	**Range**
Hospital stay (days)	7	3-15
Change in weight (kg)	2.2	0-8.9
Daily urinary output min (ml)	1495	200-4900
Systolic BP max (mmHg)	138	110-204
Systolic BP min (mmHg)	120	86-162
Platelet count min ($x10^9$/l)	70	14-292
Leukocyte count max ($x10^9$/l)	11.4	8.0-19.3
Hematocrit min	0.36	0.25-0.43
Hematocrit max	0.45	0.29-0.55
Creatinine max (µmol/l)	294	78-950
CRP max (mg/l)	96	13-269

Abbreviations: min=minimum, max=maximum, CRP=C-reactive protein, change in weight = difference between the highest and the lowest weight during hospital care (reflects fluid accumulation during the oliguric phase).


160
150
140
130
120
110
100
90
Length of spleen (mm)
Acute phase
Control phase

Plasma pentraxin-3 and coagulation and fibrinolysis variables during acute Puumala hantavirus infection and associated thrombocytopenia

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Thrombocytopenia and altered coagulation characterize all hantavirus infections. To further assess the newly discovered predictive biomarkers of disease severity during acute Puumala virus (PUUV) infection, we studied the associations between them and the variables reflecting coagulation, fibrinolysis and endothelial activation. Nineteen hospital-treated patients with serologically confirmed acute PUUV infection were included. Acutely, plasma levels of pentraxin-3 (PTX3), cell-free DNA (cf-DNA), complement components SC5b-9 and C3 and interleukin-6 (IL-6) were recorded as well as platelet ligands and markers of coagulation and fibrinolysis. High values of plasma PTX3 associated with thrombin formation (prothrombin fragments F1+2; $r = 0.46$, $P = 0.05$), consumption of platelet ligand fibrinogen ($r = -0.70$, $P < 0.001$) and natural anticoagulants antithrombin (AT) ($r = -0.74$, $P < 0.001$), protein C ($r = -0.77$, $P < 0.001$) and protein S free antigen ($r = -0.81$, $P < 0.001$) and a decreased endothelial marker ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13) ($r = -0.48$, $P = 0.04$). Plasma level of AT associated with C3 ($r = 0.76$, $P < 0.001$), IL-6 ($r = -0.56$, $P = 0.01$) and cf-DNA ($r = -0.47$, $P = 0.04$). High cf-DNA coincided with increased prothrombin fragments F1+2 ($r = 0.47$, $P = 0.04$). Low C3 levels reflecting the activation of complement system through the alternative route predicted loss of all natural anticoagulants (for protein C $r = 0.53$, $P = 0.03$ and for protein S free antigen $r = 0.64$, $P = 0.004$). Variables depicting altered coagulation follow the new predictive biomarkers of disease severity, especially PTX3, in acute PUUV infection. The findings are consistent with the previous observations of these biomarkers also being predictive for low platelet count and underline the cross-talk of inflammation and coagulation systems in acute PUUV infection. *Blood Coagul Fibrinolysis* 25:612–617 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Blood Coagulation and Fibrinolysis 2014, **25**:612–617

Keywords: cell-free DNA, coagulation, hemorrhagic fever with renal syndrome, pentraxin-3, platelet, Puumala hantavirus

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Received 8 August 2013 **Revised** 21 February 2014
Accepted 28 February 2014

Introduction

Hantaviruses cause two types of diseases, hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome in the Americas. Approximately 150 000 HFRS cases are recorded annually worldwide, and in most severe forms, the mortality rate is up to 15% [1,2]. The most common European hantavirus, Puumala virus (PUUV), causes a low mortality rate of 0.1%, but on the average, hospital care of 7 days is needed acutely, and there are long-term consequences involved, such as a need of hormone-replacement therapy and hypertension [3–5]. In turn, hantavirus cardiopulmonary syndrome with a low incidence has a case fatality rate as high as 35–50% [6–8]. Hantavirus infections are recognized as a growing public health problem, and currently there is no approved therapy or prophylaxis available [2].

The clinical hantaviral disease and its severity varies, but thrombocytopenia and altered vascular permeability are characteristics of all hantavirus infections. The mechanism of thrombocytopenia has not been fully resolved. Increased thrombin formation together with remarkable fibrinolysis and decreased natural anticoagulants have been documented [9]. Platelet ligands von Willebrand factor (VWF), fibrinogen and fibronectin are also all altered [10], these findings support the role of activated platelets and endothelium in the pathogenesis of hantavirus infection. Polymorphism of the major regulator of fibrinolysis, plasminogen activator inhibitor 1, has been found to associate with adverse kidney outcome in PUUV-HFRS [11], but there are no data on the plasma level and role of plasminogen activator inhibitor 1 in hantavirus infection. Another finding linking the coagulation system with the renal outcome

0957-5235 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI:10.1097/MBC.0000000000000117

in hantavirus diseases is that of low platelet count predicting severe acute kidney injury [12,13].

As the disease burden of hantavirus is considerable and there is no specific therapy available, biomarkers selecting the patients with the need of an early and intensive supportive treatment are of clinical value. Pentraxin-3 (PTX3) is a long pentraxin produced at the site of inflammation, and high plasma PTX3 levels have been found to associate with overall disease severity, especially thrombocytopenia, in PUUV-HFRS [14]. Cell-free DNA (cf-DNA) found in the plasma of patients with PUUV-HFRS could originate from apoptotic or necrotic cells [15], or it could be released during neutrophil activation [neutrophil extracellular traps (NETs)] [16]. High plasma cf-DNA levels have been found to associate with both the overall disease severity and low platelet count in PUUV-HFRS [17]. Complement activation as measured by the levels of complement proteins SC5b-9 and C3 has been noted to reflect the disease severity as well as thrombocytopenia in PUUV-HFRS [18]. These are all observations indicating the intimate communication of inflammation and coagulation systems in hantavirus infections.

As hantavirus infections are known for their bleeding tendency, we sought to evaluate the recently discovered predictive markers (PTX3, cf-DNA, C3 and SC5b-9 and interleukin-6 (IL-6) by studying their associations with markers of coagulation and fibrinolysis. We also discuss the possible mechanisms of thrombocytopenia related to these predictive biomarkers.

Patients and methods

The collection of clinical data, including the routine laboratory measurements, is described in detail elsewhere [9,10]. Briefly, 19 patients (17 men, median age 38 years, ranging from 30 to 64 years) were chosen of those who participated in a previous larger prospective study based on the availability of the laboratory samples. All patients suffered from serologically confirmed acute PUUV-infection [19]. They came from the Pirkanmaa area and were hospitalized in Tampere University Hospital for the median time of 7 (range 4–15) days during the period from September 2000 to December 2002. None of the patients used immunosuppression or anticoagulation, two were under antiplatelet (aspirin) therapy.

To assess the coagulation and fibrinolysis, three samples were collected in the acute phase of the disease. The first sample was taken on admission, as early as possible, 2–9 (median 6) days after the onset of fever. The second sample was drawn 3–10 (median 7) days and the third sample 6–13 (median 10) days after the onset of fever [9,10]. Laboratory analysis of plasma activated partial thromboplastin time [(APTT); Actin FSL, Siemens Healthcare Diagnostics, Marburg, Germany)], protein S free antigen level (automated latex ligand immunoassay by Instrumentation Laboratory, Lexington, Massachusetts, USA) and the antithrombin (AT) and protein C activities (both measured by chromogenic assays, BerichromAntithrombin III and BerichromProtein C, respectively, Siemens Healthcare Diagnostics) were all performed using automated coagulation instrument (BCS XP, Siemens Healthcare System, Marburg, Germany). D-dimer (an immunoturbidimetric assay Tina-quant D-Dimer, Roche Diagnostics, Mannheim, Germany) and prothrombin fragments (F1+2, a monoclonal enzyme immunoassay Enzygnost F1+2, Siemens Healthcare Diagnostics) were also assessed. The activity of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13) was determined by immunochemical detection of its proteolytic target that is VWF (Technozym Elisa; Technoclone, Vienna, Austria). The samples for plasma IL-6 measurements were collected on three consecutive mornings starting on the first morning of hospital stay, and the concentrations were determined by an enzyme-linked immunosorbent assay (PeliKine Compact human IL-6 kit; Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) [10].

All 19 patients were also participants of larger studies determining complement activation [18], PTX3 [14] and cf-DNA levels [17] during acute PUUV-infection. Blood samples for the measurements of plasma SC5b-9 and C3 were drawn four (range 2–6) times, the first sample being taken 3–7 (median 6) days after the onset of fever. Blood samples for the analyses of PTX3 (median number 4, range 3–5) and cf-DNA (median number 3, range 3–5) were all collected up to 5 consecutive days after hospitalization. The plasma PTX3 concentration was determined by an immunoassay (Quantikine; R&D Systems Inc., Minneapolis, Minnesota, USA) [14]. The plasma cf-DNA level was measured using a fluorescence-based (a DNA-intercalating dye) Quant-iT DNA High-Sensitivity Assay kit and a Qubit fluorometer (Invitrogen, Carlsbad, California, USA) [17]. The plasma C3 levels were measured by nephelometry (Dade Behring, Marburg, Germany) and SC5b-9 by an enzyme-linked immunosorbent assay (Quidel, San Diego, California, USA) [18].

The study was carried out in Tampere University Hospital, Finland and was approved by the Ethics Committee of Tampere University Hospital.

Statistical analyses

To analyze the data, the most abnormal value of each continuous variable measured during the acute phase of the disease was designated the maximum or minimum value. Medians (ranges) were provided. Relationships between the continuous variables were examined using Spearman rank correlation coefficient. The limit of significance was set at 0.05 (two-tailed). SPSS version 14.0 (Chicago, Illinois, USA) was used for computation.

Results

As reported previously, all 19 patients presented with clinical characteristics typical of PUUV-HFRS [9]. Bleeding problems were few and minor: two patients had epistaxis and one had minor conjunctival bleeding. One patient suffered from oozing at the base of the central venous catheter for several days and received a platelet transfusion of two units. No thrombotic problems occurred. The values for coagulation variables during the acute phase of the disease have been previously reported in detail [9,10]. Three patients needed transient hemodialysis treatment. All patients recovered. Table 1 presents the results of the basic laboratory tests and the predictive biomarkers of the patients.

Several associations between the coagulation variables and the predictive biomarkers could be demonstrated. Most remarkably, plasma PTX3 level associated with several variables measuring thrombin formation, coagulation and endothelial activity (Table 2). Plasma AT level was low and associated with all the predictive biomarkers (Fig. 1). Particularly strong correlations prevailed between minimum AT and minimum C3 (Fig. 1a, $r=0.76$, $P<0.001$) and between minimum AT and maximum PTX3 (Fig. 1b, $r=-0.74$, $P<0.001$). Correlations were also evident between minimum AT and maximum IL-6 (Fig. 1c, $r=-0.56$, $P=0.01$) and minimum AT and maximum cf-DNA (Fig. 1d, $r=-0.47$, $P=0.04$). Interestingly, D-dimer did not associate with any of the predictive biomarkers studied here.

Plasma cf-DNA associated with the screening test for coagulation that is APTT and the formation of prothrombin fragments F1+2 ($r=0.47$, $P=0.04$ and $r=0.49$, $P=0.03$, respectively). Associations between cf-DNA levels and markers of complement activation SC5b-9 and C3 were also noted ($r=0.61$, $P=0.01$ and $r=-0.55$, $P=0.02$, respectively).

Plasma C3 levels were found to associate with APTT ($r=-0.70$, $P=0.003$). A negative correlation prevailed between the plasma levels of C3 and natural anticoagulants protein C activity and protein S free antigen ($r=0.53$, $P=0.03$ and $r=0.64$, $P=0.004$, respectively).

Table 1 Basic laboratory values and predictive biomarkers in 19 patients with acute Puumala hantavirus infection

	Median (range)	Reference
Platelet count min ($\times 10^9$/l)	75 (13–238)	150–360
Creatinine max (μmol/l)	321 (74–1285)	<105 m, <95 f
CRP max (mg/l)	61 (6–198)	<10
Interleukin-6 max (pg/ml)	16.7 (3.6–96.6)	<0.4
SC5b-9 max (ng/ml)	595 (170–1034)	–
C3 min (g/l)	1.2 (0.4–2.1)	–
cf-DNA max (μg/ml)	1.3 (1.1–3.3)	–
PTX3 max (ng/ml)	91 (8–1251)	–

C3, complement protein C3; cf-DNA, cell-free DNA; CRP, C reactive protein; f, females; m, males; max, maximum; min, minimum; PTX3, pentraxin-3; SC5b-9, complement protein SC5b-9.

Table 2 Associations of plasma pentraxin-3 levels and markers of coagulation in 19 patients with acute Puumala hantavirus infection

Plasma pentraxin-3	*r*	*P*
APTT max	0.75	<0.001
Thrombin time min	−0.61	0.005
Fibrinogen min	−0.70	<0.001
Antithrombin min	−0.74	<0.001
Protein C activity min	−0.77	<0.001
Protein S free antigen min	−0.81	<0.001
Prothrombin fragments F1+2 min	0.46	0.05
ADAMTS13 min	−0.48	0.04

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13; APTT, activated partial thromboplastin time; max, maximum; min, minimum.

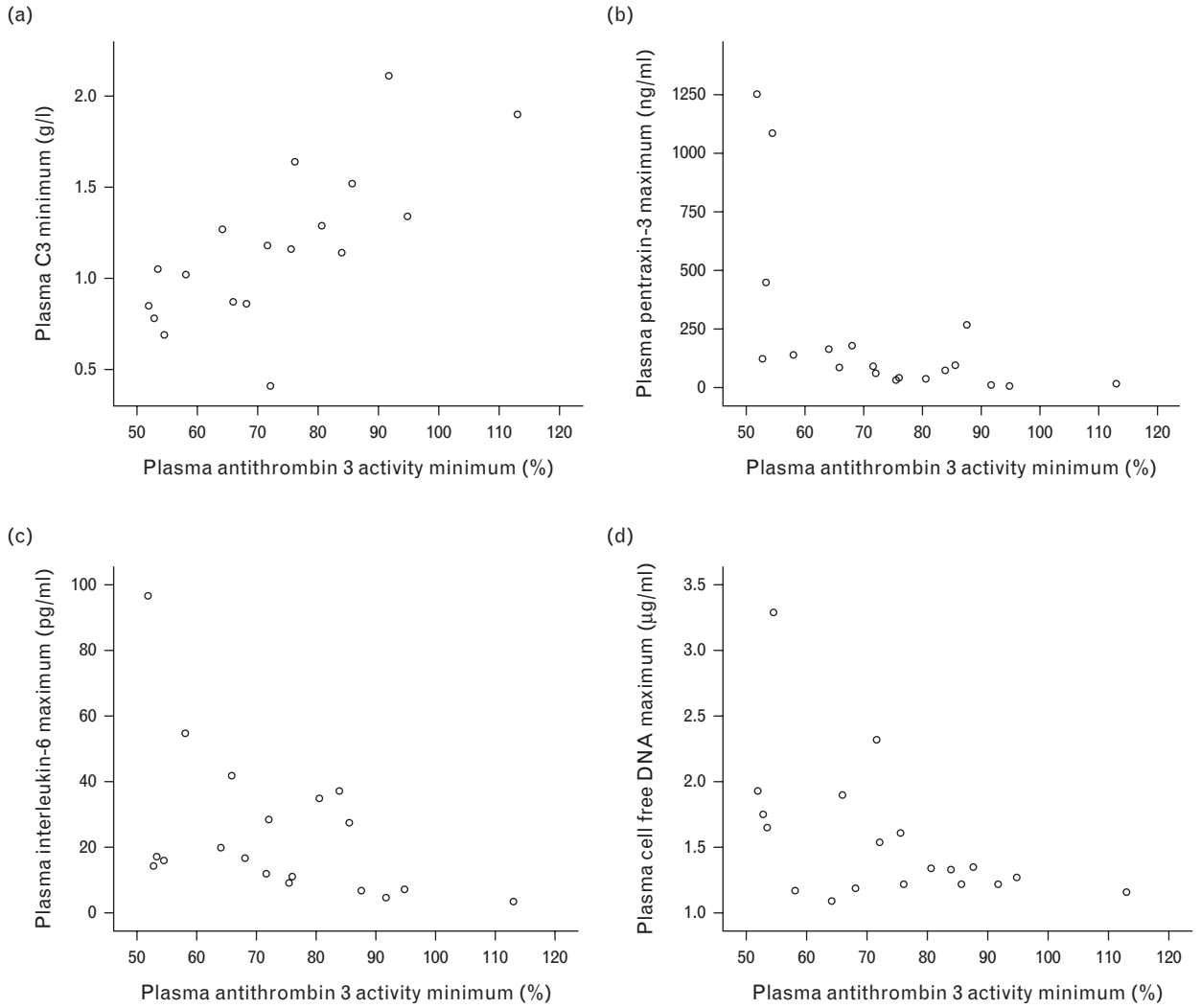
Discussion

We here studied the associations of PTX3, cf-DNA, C3 and SC5b-9 and IL-6 with the platelet ligands and the variables measuring endothelial activation, coagulation and fibrinolysis during acute PUUV infection. Our aim was to further assess the relevance of these biomarkers in the prediction of disease severity in this viral infection known for the clinical bleeding tendency. We also sought to discuss some of the possible mechanisms leading to the decreased platelet count typical for all hantaviral diseases. Several associations between the predictive biomarkers and hemostatic variables could be detected. The most remarkable observation was the association of plasma PTX3 level with several variables depicting activation of endothelium and coagulation, a finding emphasizing the relevance of PTX3 in the prediction of disease severity in PUUV infection. The association of low natural anticoagulant AT with high values of all biomarkers predicting severe disease was notable.

PTX3 is an acute-phase reactant belonging to the family of pentraxins. The long PTX3 is produced in peripheral tissues and monocytic macrophages in response to pro-inflammatory cytokines IL-1 and tumor necrosis factor, and toll-like receptor agonists [20–22]. The short pentraxins C-reactive protein and serum amyloid P are induced in hepatocytes in response to IL-6 [23]. Pentraxins opsonize pathogens or apoptotic cells for their rapid clearance by phagocytes or induce pathogen killing in extracellular compartments [24]. High PTX3 has been found to associate with various clinical conditions indicating adverse prognosis. As for PTX3 and viral infections, studies are few but include one indicating that PTX3 concentrations are higher in patients suffering from dengue shock syndrome than in patients with dengue fever or dengue hemorrhagic fever [25]. High PTX3 is also known to predict severe disease and low platelet count in acute PUUV infection [14].

Unlike the long PTX3, the short pentraxin C-reactive protein does not reflect the clinical severity of PUUV infection [26], but high C-reactive protein associates with low AT and protein C [9]. In the present study, high PTX3 was found to associate with several variables

Fig. 1



Associations between plasma antithrombin and (a) complement protein C3, (b) pentraxin-3, (c) interleukin-6 and (d) cell-free DNA in 19 patients with acute Puumala hantavirus infection.

reflecting thrombin formation and activation of coagulation system and endothelium. The correlations were especially strong between high levels of PTX3 and low levels of natural anticoagulants AT, protein S free antigen and protein C. PTX3 is known to increase tissue factor expression by mononuclear cells, which results in thrombin formation, clotting activation and loss of natural anticoagulants [27]. PTX3 also facilitates C1q-mediated activation of the classical complement pathway [28] and interacts with P-selectin [29,30]. These are all mechanisms possibly providing links between increased PTX, activated coagulation and loss of platelets. The association of high PTX3 with both severity of clinical disease [14] and variables reflecting activation of endothelium and coagulation suggests that PTX3 could be determined when initially evaluating a patient with acute PUUV infection.

AT inhibits several serine proteases critical to the regulation of coagulation, most importantly thrombin and activated coagulation factor X [31]. Furthermore, AT has shown a variety of anti-inflammatory actions independent of its anticoagulatory effects. AT inhibits leukocyte activation in part due to the release of prostacyclin and the downregulation of P-selectin [32]. The anti-inflammatory mechanisms of AT also rely on its binding to heparin sulfate proteoglycan on the endothelial cell surface [33]. Proinflammatory cytokines, such as IL-6, decrease the production of endothelial heparin sulfate proteoglycans and may thus contribute to the diminished anti-inflammatory effect of AT in hantavirus infections as well as in other systemic inflammatory response syndromes [34]. Reduced plasma levels of AT may be caused by decreased liver production, degradation by elastase from activated neutrophils [35] and

consumption as a consequence of ongoing thrombin formation, the latter reported to occur during acute hantavirus infection [9]. Previously, low plasma levels of AT have been observed together with low platelet count during acute PUUV infection [9]. In this study, low plasma levels of AT were found to associate with high plasma levels of PTX3, cf-DNA, IL-6 and low plasma C3 during acute PUUV infection. These are all biomarkers recently shown to predict severe disease depicted by low platelet count, high leukocyte count and longer need for hospital care [14,17,18,26]. The observation of low plasma AT associating with biomarkers predicting severe disease in hantavirus infection is in line with the reports of associations between severity of disease and degree of AT depletion in the setting of sepsis and critically ill patients [33,35,36]. As for the AT replacement therapy, the clinical benefits have been found unsure and could possibly be achieved only if large supraphysiological doses are used [36]. In order to measure the plasma AT level as a part of the initial evaluation of a patient with acute hantavirus disease, more conclusive evidence is needed on the association between the AT level and the severity of clinical disease.

Increased fibrinolysis indicated by very high level of plasma D-dimer has been recorded during acute PUUV infection [9,37], and high plasma levels of D-dimer have been observed in severe PUUV infections [37]. In dengue virus infection, high D-dimer associates with proinflammatory cytokine tumor necrosis factor, and increased plasminogen conversion to plasmin induced by dengue virus antibodies is suggested [38]. We could not find any associations between D-dimer and PTX3, cf-DNA, S-C5b-9, C3 or IL-6, all biomarkers previously found to predict severe clinical disease and low platelet count in PUUV infection. This is a finding well in accordance with our previous observation of D-dimer not being predictive of disease severity in this same study population [9]. So far, our observations do not support the measurement of D-dimer as a predictive biomarker in the initial evaluation of a patient with acute PUUV infection.

The plasma levels of cf-DNA are reported to be high and associate with severe disease and low platelet count in acute PUUV infection [17]. Apoptotic cells are a possible source of extracellular DNA [39], even though no direct viral cytopathic effect has been detected in hantavirus infections. Another cause for the extracellular DNA could be the formation of NETs as a part of innate immune response to inflammation. Histones appear to be the dominant component of the NETs leading to coagulation [40]. They promote thrombin formation through platelet-dependant mechanisms [41,42] and impair the protein C pathway [43] thereby shifting the hemostatic balance to the procoagulant direction. In this study, high cf-DNA levels are associated with thrombin formation, loss of AT and complement activity. However, no thrombotic events occurred in the present study population, which are compatible with previous observations in hantavirus diseases. The concept of NET formation during acute PUUV infection bears scientific interest, but measurements of plasma cf-DNA are not yet a tool for the clinical evaluation in this group of patients.

In this study, the associations between C3 and APTT and natural anticoagulants AT, protein C and protein S free antigen indicated ongoing simultaneous activity in the alternative pathway of the complement system and blood coagulation during acute PUUV infection. These results are in line with the previous reports establishing associations between decreased C3 levels and coagulation factors in Hantaan virus-induced HFRS [44] and with the knowledge of the extensive intercommunication between the complement system and blood coagulation and fibrinolysis [45]. Activation of the complement system has been stated a frequent finding in PUUV-induced HFRS [18,46], and the disease severity has been associated both with the classical [46] and the alternative route [18] activation. Thus, far measurements of complement activation have not provided substantial benefit in the clinical evaluation of patients with acute PUUV infection.

A limitation of this study is a relatively small number of patients included. Further, the findings show only associations between the different variables in patients with acute PUUV infection. A comparison of these results with those obtained from control patients diagnosed with other acute viral infections would assess the specificity of these findings for acute PUUV infection. There are also a number of variables, such as low specific immunoglobulin G titer, that potentially predict severe disease [47] but are not included in this study. However, in this study, the biomarkers recently discovered to predict disease severity in PUUV infection were consistently found to associate with variables reflecting thrombin formation and coagulation, and potential molecular mechanisms between the inflammation and coagulation systems that lead to thrombocytopenia could be discussed.

We conclude that measuring plasma PTX3 as a part of the initial evaluation of patients with acute PUUV infection could help to earlier find those with the most severe disease.

Acknowledgements

The skillful technical assistance of Mirja Ikonen, Katriina Yli-Nikkilä and Eini Eskola is greatly appreciated.

Sources of support: the Blood Disease Research Foundation, the Finnish Association of Hematology, the Finnish Kidney Foundation and Sigrid Jusélius Foundation.

Conflicts of interest

There are no conflicts of interest.

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Diminished coagulation capacity assessed by calibrated automated thrombography during acute Puumala hantavirus infection

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Coagulation abnormalities are associated with Puumala-virus-induced hemorrhagic fever with renal syndrome (PUUV–HFRS). We evaluated the coagulation capacity of plasma during acute PUUV–HFRS by measuring thrombin generation using calibrated automated thrombography (CAT). The study cohort comprised 27 prospectively collected, consecutive, hospital-treated patients with acute PUUV infection. Blood samples were drawn in the acute phase and at the control visit approximately 5 weeks later. To evaluate thrombin generation, the lag time of initiation, endogenous thrombin potential (ETP), and peak and time to peak thrombin concentration were assessed by CAT in platelet poor plasma without corn trypsin inhibitor. Plasma levels of D-dimer, fibrinogen and prothrombin fragments (F1 + 2) were also evaluated. When the acute phase was compared with the control phase, ETP was decreased (median 1154 nmol/l/min, range 67–1785 vs. median 1385 nmol/l/min, range 670–1970; $P<0.001$), while the lag time was prolonged (median 3.8 min, range 2.1–7.7 vs. median 2.9 min, range 2.0–4.1; $P<0.001$). Low ETP correlated with low peak thrombin concentration ($r=0.833$, $P<0.001$). Prolonged time to peak associated with the lag time ($r=0.78$, $P<0.001$). ETP was associated with thrombocytopenia ($r=0.472$, $P=0.015$) and weakly with fibrinogen level ($r=0.386$, $P=0.047$). The measured CAT parameters did not associate with D-dimer and F1 + 2 levels. Decreased ETP together with low peak and prolonged lag time indicate decreased plasma potential for thrombin generation *in vitro*. Together with low platelet count and enhanced fibrinolysis, this further refers to altered blood coagulation and increased propensity toward bleeding in acute PUUV–HFRS. *Blood Coagul Fibrinolysis* 29:55–60

Blood Coagulation and Fibrinolysis 2018, **29**:55–60

Keywords: calibrated automated thrombography, coagulation, fibrinolysis, hantavirus, platelet, thrombin

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Received 6 July 2017 **Revised** 30 August 2017
Accepted 12 September 2017

Introduction

Hantaviruses are the cause of two disease entities: hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus cardiopulmonary syndrome in North and South America. Puumala hantavirus (PUUV) causes mild HFRS, also called nephropathia epidemica in Europe [1,2]. PUUV–HFRS is characterized by thrombocytopenia and coagulation abnormalities, acute kidney injury and capillary leakage [1–3]. Petechiae, epistaxis, hematuria and conjunctival bleedings are common [1]. Hemorrhagic gastropathy is observed in all PUUV–HFRS patients in gastroscopy [4]. Severe and fatal hemorrhages of pituitary gland, kidneys, heart, liver, lungs and peritoneal cavity have been described [5,6]. The risk for cardiovascular disease has also been linked with PUUV–HFRS [7]. Disseminated intravascular coagulation has been encountered in severe cases [8,9].

Hantaviruses target vascular endothelial cells via ß3 integrin receptor and adhere quiescent platelets to the endothelium, thus contributing to vascular permeability and thrombocytopenia [10]. Through interactions with hantavirus, platelets and endothelium, alterations in the coagulation system occur. Previous studies imply enhanced thrombin formation, as evaluated by shortened prothrombin time (PT) and thrombin time (TT), and overall increase in prothrombin fragments 1 + 2 (F1 + 2), and decreased levels of natural anticoagulants, antithrombin (AT), protein C (PC) and protein S free antigen [8,11]. A study with PUUV-infected human umbilical vein endothelial cells suggests increased tissue factor (TF) activity [12]. Fibrinolysis is activated as indicated by increased concentrations of fibrin degradation products, D-dimer and tissue plasminogen activator (tPA) [8,13]. Platelet ligands are altered, and ADAMTS13 activity is decreased [14].

Thrombin is the key enzyme during coagulation leading to the conversion of fibrinogen to fibrin and clot formation. Thrombin generation assays are useful indicators of the

DOI:10.1097/MBC.0000000000000667

overall plasma coagulability, in contrast to the conventional coagulation tests that mainly assess individual factors or a part of the coagulation pathway. Calibrated automated thrombography (CAT) measures in-vitro thrombin generation of plasma by continuous cleavage of a fluorogenic substrate, thus expressing the overall haemostatic potential [15]. CAT is applied in research of vascular thrombosis, bleeding disorders and monitoring of anticoagulant treatment [16]. To our knowledge, studies on thrombin generation by CAT in hantavirus infections are yet lacking.

Both hemorrhagic and thrombotic events have been associated with PUUV–HFRS, but the underlying mechanisms of alterations in coagulation system are not well defined. Therefore, we aimed to evaluate the plasma coagulation capacity in PUUV-infected patients by measuring thrombin generation by CAT. The goal was to describe how thrombin generation is altered during the acute phase of infection, and further determine the possible hypocoagulability or hypercoagulability associated with hantavirus infection. We also sought to investigate the possible associations of CAT assay with the tests applied to measure thrombin formation and fibrinolysis in clinical practice and variables depicting disease severity of acute PUUV–HFRS.

Material and methods

Ethics statement

All patients were recruited and enrolled after providing a written informed consent. The study protocol was approved by the Ethics Committee of Tampere University Hospital. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Patients

The study was carried out in Tampere University Hospital, University of Tampere, Helsinki University Hospital and University of Helsinki. All patients came from the Pirkanmaa area and were hospitalized at Tampere University Hospital due to serologically confirmed acute PUUV–HFRS [17] during the period from October 2010 to February 2014.

Twenty-seven prospectively collected, consecutive patients (17 men) with acute PUUV–HFRS were included. Their median age was 50 years (range 21–67 years). None of the patients used anticoagulant or immunosuppressive therapy. Two patients used an antiplatelet drug (acetylsalicylic acid).

Clinical and laboratory data

The following variables were recorded: the number of days from the onset of fever before the acute-phase study samples were collected, the length of hospital stay (days), signs of bleeding symptoms (yes/no), thromboembolic complications (yes/no), need for transient hemodialysis treatment (yes/no) and maximum gain in weight (kg). Complete blood count, plasma C-reactive protein (CRP) and plasma creatinine were measured according to clinical need. The laboratory analyses were carried out at the Laboratory Centre of Pirkanmaa Hospital District using standard methods.

Methods

The study design was longitudinal with two time-points of blood draw for CAT. The acute phase samples ($n = 27$) were taken median 7 days (range 4–12 days) from the onset of fever. Control samples ($n = 23$) were taken at the follow-up visit, median 43 days (range 38–76 days) from the onset of fever. The blood count was assessed in the acute and control phase of CAT study days, and the lowest platelet count during the hospital stay was recorded.

CAT analyses and plasma measurements of fibrinogen, F1 + 2 and D-dimer were carried out in Clinical Chemistry coagulation laboratory (HUSLAB Laboratory Services, Helsinki University Central Hospital, Finland). D-Dimer (Tina Quant D-Dimer; Roche Diagnostics, Mannheim, Germany) and fibrinogen (Multifibren U; Siemens Healthcare Diagnostics Marburg, Germany) levels were determined according to manufacturer's recommendations. F1 + 2 were measured by an enzyme immunoassay (Enzygnost F1 + 2, monoclonal; Siemens Healthcare Diagnostics). The reference values for D-dimer were 0.5 mg/l or less, fibrinogen 1.7–4.0 g/l and F1 + 2 69–229 pmol/l.

Measurement of thrombin generation by calibrated automated thrombography

Thrombin generation was measured using CAT (Diagnostica Stago, Thrombinoscope BV, Maastricht, the Netherlands) with the Stago platelet poor plasma (PPP) reagent (TF 5 pmol/l and phospholipids 4 μmol/l) without corn-trypsin inhibitor. The lag time of the initiation of thrombin generation (lag time, min), the endogenous thrombin potential (ETP; the area under the curve; nmol/l thrombin × time), peak (maximum thrombin concentration, nmol/l) and time to peak (tt Peak, min) were measured and recorded according to the manufacturer's instructions. Blood samples were collected into sodium citrate anticoagulant (3.2%; 109 mmol/l) tubes according to the local sampling protocol as part of hospital routine and centrifuged (at 2500 × g for 15 min). The PPP was separated within 2 h and stored at −80 °C before analysis.

Statistics

All continuous, skewed variables were determined as medians and ranges. The associations between thrombin generation markers and clinical and laboratory variables were evaluated for continuous data by Spearman rank correlation coefficient. To analyze the changes between the acute and control phase, Wilcoxon-signed rank test was used for pairwise comparisons. The level of significance was set at P value 0.05 (2-tailed). Statistical analyses were performed with IBM SPSS Statistics for Windows version 22.0 (Armonk, New York, USA).

Table 1 The clinical and laboratory findings during hospital care in 27 patients with acute Puumala hantavirus infection

Clinical or laboratory variable	Median	Range
Days from the onset of illness[a]	7	4–12
Length of hospital stay (days)	7	3–12
BMI (kg/m^2)	26.6	22.3–36.8
Change in weight (kg)[b]	3.8	0.5–11.3
SBP min (mmHg)	108	80–135
DBP min (mmHg)	67	55–83
Creatinine max (μmol/l)	268	71–983
Leukocyte count max ($\times 10^9$/l)	10.7	4–45
Hemoglobin max (g/l)	155	122–214
Hematocrit max	0.43	0.37–0.60
Platelet count min ($\times 10^9$/l)	60	5–150
CRP max (mg/ml)	79	21–244

Reference values: hematocrit 0.35–0.50 for men and 0.35–0.46 for women, platelet count 150–360, leukocyte count 3.4–8.2 $\times$ 10^9/l, CRP less than 10 mg/ml, creatinine less than 105 μmol/l for men and less than 95 μmol/l for women. BP, blood pressure; CRP, C-reactive protein; max, maximum; min, minimum; PUUV–HFRS, Puumala virus-induced hemorrhagic fever with renal syndrome. [a]The number of days of fever before the first study samples were obtained. [b]Reflects fluid accumulation in the oliguric phase of PUUV–HFRS.

Results

Clinical and laboratory findings

All 27 patients suffered from clinically typical and serologically confirmed PUUV–HFRS [17]. The clinical and laboratory findings of the patients are shown in Table 1. Mild bleedings were reported in eight patients including nasal and conjunctival hemorrhages, petechiae, hemoptysis and melena. There were no thromboembolic events recorded. None of the patients needed transient hemodialysis treatment.

The acute phase median platelet count was 68×10^9/l (range $8–222 \times 10^9$/l), CRP 57 mg/ml (10–178 mg/ml), hemoglobin 139 g/l (120–177 g/l), hematocrit 0.40 (0.34–0.49) and creatinine 126 μmol/l (52–699 μmol/l). Twenty-four out of 27 patients were thrombocytopenic (lowest platelet count $<150 \times 10^9$/l).

Thrombin generation by calibrated automated thrombography

When compared with the control phase, ETP was diminished by 16% (1154, 67–1785 vs. 1385, 670–1970 nmol/l/min; $P < 0.001$). In addition, tt peak was prolonged (7.3, 4.8–14.9 vs. 5.9, 4.3–9.8 min; $P = 0.012$). Peak thrombin concentration was lowered (204, 5.6–293 vs. 243, 106–331 nmol/l; $P = 0.008$), and lag time was prolonged (3.8, 2.1–7.7 vs. 2.9, 2.0–4.1 min; $P < 0.001$) in the acute phase. Accordingly, ETP correlated with peak thrombin concentration ($r = 0.833$, $P < 0.001$; Fig. 1a). Tt peak associated with the lag time ($r = 0.78$, $P < 0.001$; Fig. 1b). An inverse correlation was observed between peak and tt peak ($r = -0.54$, $P = 0.004$).

Associations of calibrated automated thrombography parameters with variables depicting clinical disease

In the acute phase, ETP associated with the platelet count measured in the CAT study day sample ($r = 0.472$, $P = 0.015$; Fig. 1c), and with the lowest platelet count measured during the hospital stay ($r = 0.402$, $P = 0.038$). Similarly, peak thrombin concentration associated with the platelet count of the CAT study day ($r = 0.554$, $P = 0.003$), and with the lowest platelet count measured during the hospital stay ($r = 0.462$, $P = 0.015$). Prolonged lag time and low ETP associated with increased fibrinogen level measured in the acute phase ($r = 0.511$, $P = 0.006$ and $r = 0.386$, $P = 0.047$, respectively; Fig. 1d). The fibrinogen level was acutely increased compared with the control phase (median 4.2, range 2.2–9.6 g/l and median 3.4 g/l, range 2.6–4.9 g/l, respectively; $P = 0.005$).

D-Dimer was increased in the acute phase (2.8, 0.6–34 vs. 0.4, 0.2–1.1 mg/l; $P < 0.001$). D-Dimer associated strongly with F1 + 2 ($r = 0.843$, $P < 0.001$). F1 + 2 was increased acutely (704, 284–1875 vs. 263, 118–556 pmol/l; $P < 0.001$). ETP did not associate with D-dimer and F1 + 2 ($r = -0.079$, $P = 0.695$ and $r = -0.164$, $P = 0.415$, respectively). There were no associations between peak and D-dimer and F1 + 2 ($r = -0.030$, $P = 0.882$ and $r = -0.162$, $P = 0.418$, respectively).

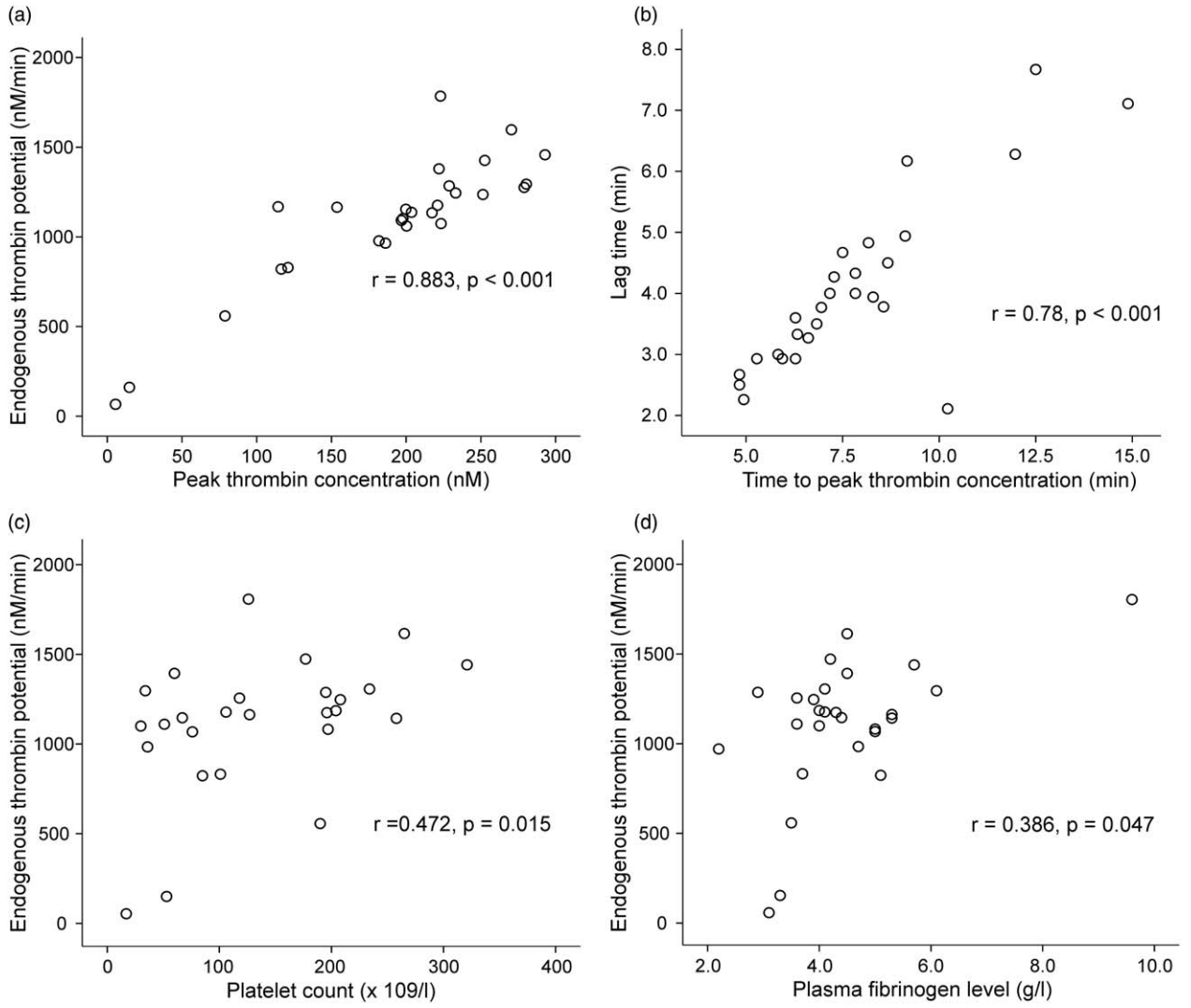
Clinical variables depicting the disease severity, that is maximum leukocyte count, maximum plasma creatinine, CRP level and length of hospital stay, were not associated with CAT parameters (data not shown). Furthermore, reduced ETP was not associated with bleedings (data not shown).

Discussion

The primary aim of this study was to investigate whether thrombin generation, measured using CAT assay, is altered during acute PUUV–HFRS. In addition, we sought to elucidate the underlying coagulation mechanisms predisposing to bleeding and thrombosis. The main findings were reduced ETP and peak thrombin concentration suggesting diminished plasma potential for thrombin generation during acute PUUV–HFRS. Prolonged lag time indicating slower initiation of burst, and extended time to reach the peak representing the velocity of thrombin generation, further support the finding of hypocoagulability. Decreased platelet count, one of the clinical characteristics of hantavirus infection, was found to associate with low ETP and low peak thrombin concentration. We did not find statistically significant associations between prothrombin fragments F1 + 2 (F1 + 2), D-dimer and CAT parameters in this study population.

Previous studies imply enhanced thrombin generation in acute PUUV–HFRS on the basis of increased prothrombin fragments F1 + 2 generated during conversion of prothrombin to thrombin, and an increased level of fibrin degradation product D-dimer [8,9,11]. High levels of circulating F1 + 2 and D-dimer observed in this study confirm previous findings. In-vivo thrombin generation parameters, F1 + 2 and D-dimer, strongly depend on the amount of TF and thrombomodulin present on vascular

Fig. 1



Scatter plots illustrating the correlation between endogenous thrombin potential and peak thrombin concentration (a) lag time and time to peak thrombin concentration (b), endogenous thrombin potential and simultaneous platelet count (c) and endogenous thrombin potential and plasma fibrinogen level (d) during acute Puumala virus infection.

endothelial cells [16,18]. In-vivo thrombin generation is also ongoing in the microparticles released during infection [19,20]. Data suggest increased TF in endothelial cells in the acute phase of PUUV–HFRS [12]. On the other hand, the in-vitro thrombin potential assessed by CAT determines how thrombin can be generated by plasma containing a defined amount of thrombin generation trigger. Thus, it measures the haemostatic balance of plasma clotting factors and inhibitors independently of the procoagulant and inhibitory drivers released by the endothelium [18]. The difference between in-vivo and in-vitro thrombin generation is well reflected in consumption coagulopathy, a condition in which indicators of ongoing coagulation are increased, but plasma potential of thrombin generation is decreased [21].

A previous study indicates slightly prolonged PT, prolonged activated partial thromboplastin time (APTT) and shortened TT during acute PUUV–HFRS [8,18]. These in-vitro coagulation tests cannot detect the in-vivo contribution of endothelial cells and shear stress of blood flow on local clot formation and fibrinolysis. Traditional coagulation tests APTT and PT assess the time to the initiation of clot formation, and thus, they do not entirely reflect the hemostatic balance in acutely ill patients [22]. These plasma clotting assays are considered to reflect the lag time phase of thrombin generation in CAT assay [22]. Our observation of prolonged lag time indicating slower initiation of thrombin generation is in line with the previous findings of the coagulation tests APTT and PT. As the data concerning in-vitro

coagulation tests are lacking in the current study population, the direct comparison with CAT parameters is not possible.

Fibrinolysis is increased, as indicated by high D-dimer and endothelial cell tPA levels, during PUUV infection [8,11–13]. The plasminogen activator inhibitor 1 level is not altered in the acute phase [13]. Thrombomodulin-associated thrombin activates the thrombin-activatable fibrinolysis inhibitor (TAFI), which downregulates fibrinolysis. It can be speculated that low ETP may contribute to decreased TAFI and thus increased fibrinolysis and bleeding tendency [23]. Diminished thrombin generation in PUUV–HFRS together with excessive fibrinolysis also resembles the data obtained in another hemorrhagic fever, dengue virus infection [24,25]. In dengue fever, the bleeding complications have been shown to associate with reduced thrombin formation along with thrombocytopenia and enhanced fibrinolysis [26]. We did not find an association between low ETP and bleedings, although mild bleedings were reported in one third of the patients.

We are aware of the relatively small sample size of the study. Yet, the associations were statistically significant, even if the number of clinical events remained minor. The levels of coagulation factors were not available and individual acquired or inherited factors affecting hemostasis could not be assessed. Two patients used aspirin, a platelet antagonist that inhibits platelet aggregation and thrombin formation. As thrombin generation was assessed in PPP, it is unlikely that aspirin, attached to the minor amount of residual platelets, could affect these CAT results.

Ongoing in-vivo coagulation may result in consumption of platelets and coagulation factors during acute PUUV-infection. Correlation of low platelet count with low ETP and low peak may imply thrombin activation and consumption of platelets, as thrombopoiesis is shown to be active during acute PUUV–HFRS [20]. Natural anticoagulants, PC and protein-S-free antigen and AT are also found to be decreased in the acute phase of PUUV–HFRS [8]. Increased TF expression on the endothelial cells and microparticle release might result in consumption of platelets and clotting factors resulting in lower ETP. All of these findings are supported by the previous reports [8,11,20].

In conclusion, in this study, we found decreased in-vitro thrombin generation measured by CAT in acute PUUV infection. Together with thrombocytopenia, increased fibrinolysis and signs of enhanced thrombin generation *in vivo* this data suggest a mild-to-moderate consumption coagulopathy during acute PUUV–HFRS. The CAT results of plasma hypocoagulobility support previous findings of impaired hemostasis during acute PUUV–HFRS. Larger future studies might further clarify the role of coagulation in the pathogenesis of HFRS.

Acknowledgements

The skillful technical assistance of Eini Eskola, Marja Lemponen and Kati Ylinikkilä is greatly appreciated.

The study was financially supported by the Competitive State Research Financing of the Expert Responsibility Area of Tampere University Hospital (9P031), Tampere Tuberculosis Foundation, Sigrid Jusélius Foundation, Maud Kuistila Foundation, Finnish Association of Hematology and Finnish Kidney Foundation.

Conflicts of interest

There are no conflicts of interest.

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RESEARCH ARTICLE

Endothelial Nitric Oxide Synthase G894T Polymorphism Associates with Disease Severity in Puumala Hantavirus Infection

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Citation: Koskela S, Laine O, Mäkelä S, Pessi T, Tuomisto S, Huhtala H, et al. (2015) Endothelial Nitric Oxide Synthase G894T Polymorphism Associates with Disease Severity in Puumala Hantavirus Infection. PLoS ONE 10(11): e0142872. doi:10.1371/journal.pone.0142872

Editor: Joerg Latus, Robert Bosch Hospital, GERMANY

Received: August 30, 2015

Accepted: October 27, 2015

Published: November 11, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The study was financially supported by the Competetive State Research Financing of the Expert Responsibility Area of Tampere University Hospital, European Union 7th Framework Program grant number 201668 for the AtheroRemo Project, the grant NIH/NIAID U19 A157319, the Finnish Kidney Foundation, and the Maud Kuistila Foundation and Sigrid Juselius Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The

Abstract

Introduction

Hantavirus infections are characterized by both activation and dysfunction of the endothelial cells. The underlying mechanisms of the disease pathogenesis are not fully understood. Here we tested the hypothesis whether the polymorphisms of endothelial nitric oxide synthase, eNOS G894T, and inducible nitric oxide synthase, iNOS G2087A, are associated with the severity of acute Puumala hantavirus (PUUV) infection.

Patients and Methods

Hospitalized patients (n = 172) with serologically verified PUUV infection were examined. Clinical and laboratory variables reflecting disease severity were determined. The polymorphisms of eNOS G894T (Glu298Asp, rs1799983) and iNOS G2087A (Ser608Leu, rs2297518) were genotyped.

Results

The rare eNOS G894T genotype was associated with the severity of acute kidney injury (AKI). The non-carriers of G-allele (TT-homozygotes) had higher maximum level of serum creatinine than the carriers of G-allele (GT-heterozygotes and GG-homozygotes; median 326, range 102–1041 vs. median 175, range 51–1499 μmol/l; p = 0.018, respectively). The length of hospital stay was longer in the non-carriers of G-allele than in G-allele carriers (median 8, range 3–14 vs. median 6, range 2–15 days; p = 0.032). The rare A-allele carriers (*i.e.* AA-homozygotes and GA-heterozygotes) of iNOS G2087A had lower minimum systolic and diastolic blood pressure than the non-carriers of A-allele (median 110, range 74–170 vs.116, range 86–162 mmHg, p = 0.019, and median 68, range 40–90 vs. 72, range 48–100 mmHg; p = 0.003, respectively).

Fimlab Laboratories Ltd., provided support in the form of salaries for authors (ST, PJK), but did not have any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the `authors contributions` section.

Competing Interests: Sari Tuomisto and Pekka J. Karhunen are employed by Fimlab Laboratories. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

Conclusions

Patients with the TT-homozygous genotype of eNOS G894T had more severe PUUV-induced AKI than the other genotypes. The eNOS G894T polymorphism may play role in the endothelial dysfunction observed during acute PUUV infection.

Introduction

Hantaviruses cause two clinical syndromes in humans, the haemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. Puumala hantavirus (PUUV) is the most common hantavirus causing HFRS in Europe [1]. The main characteristics of PUUV-HFRS are increased capillary leakage, thrombocytopenia and acute kidney injury (AKI). Although PUUV-HFRS has a low rate of case fatality (up to 0.4%), significant acute-phase complications as well as long-term hormonal, renal and cardiovascular consequences can occur [1, 2].

The main pathophysiological mechanisms of hantavirus infection include activation of cytokines [3, 4] and cytotoxic CD8+ T-lymphocytes [5], vascular endothelial growth factors [6, 7], and the complement system [8]. Recently discovered biomarkers that reflect PUUV-HFRS disease severity include pentraxin-3, indoleamine 2,3-dioxygenase, plasma cell-free DNA, soluble urokinase-type plasminogen activator and GATA-3 [9]. Host genetic factors also influence the outcome of acute PUUV infection. In the Finnish population, individuals with Human Leukocyte Antigen (HLA) alleles B8, C4A*Q0 and DRB1*0301 are more prone to have a severe form of PUUV infection [10, 11]. Also polymorphisms of the cytokines tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and IL-1 receptor antagonist impact on the clinical severity of PUUV-HFRS [10, 12]. Likewise, genetic polymorphisms of plasminogen activator inhibitor, the main physiological regulator of fibrinolysis, and platelet glycoprotein 1a, associate with severe PUUV infection [13].

Increased nitric oxide (NO) levels induced by elevated TNF-α concentrations have been suggested to participate in the pathogenesis of hantaviral infections [14, 15, 16]. Elevated concentrations of NO correlate with increased serum creatinine value and hypotension, and inversely correlate with platelet count in patients with acute PUUV infection [15]. According to a Swedish study, NO has antiviral effects on hantaviruses by inhibiting viral replication at the early phase of infection [17]. Endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) that can be induced in a variety of cell types, are the key enzymes catalysing NO synthesis [18].

A widely explored polymorphism of the eNOS gene, the G894T (rs1799983) polymorphism encoded by the NOS3 gene in chromosome 7, has been linked to increased risk of coronary artery disease (CAD), myocardial infarction, coronary spasms, hypertension, and ischemic stroke [19–24]. Recent data suggest that the T-allele of G894T polymorphism is also associated with increased susceptibility to and risk of end-stage renal disease (ESRD) [25], and also with earlier onset age of ESRD in males with autosomal dominant polycystic disease [26]. The iNOS, encoded by the NOS2 gene in chromosome 17, is expressed in macrophages, neutrophils and hepatocytes as a host immune response to cytokines. The G2087A (rs2927518) polymorphism of iNOS has been implicated in a variety of diseases, including inflammatory bowel disease, gastric cancer, migraine with aura, septic shock, and non-Hodgkin lymphoma [27–30]. So far there is no evidence whether the NOS polymorphisms influence the clinical course of hantaviral infections.

We aimed to study the influence of the above polymorphisms that have the potential to affect endothelial and vascular function, eNOS G894T (Glu298Asp, rs1799983) and iNOS G2087A (Ser608Leu, rs2297518), on disease severity in patients with acute PUUV infection. We sought to determine whether the genetic variations within the genes of NOS3 and NOS2 could contribute to individual differences in the outcome of acute PUUV infection.

Material and Methods

Ethics statement

The study was carried out at Tampere University Hospital and University of Tampere, School of Medicine. All patients were recruited and enrolled after providing a written informed consent. In addition, informed verbal consent was obtained from the guardians of the minors. Blood samples of the minors were collected before the current Medical Reseach Act was valid in Finland. The Ethics Committee of Tampere University Hospital approved the study protocol and consent procedure according to the ethical principles at the time of the study. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Patients

The study cohort consisted of 172 prospectively collected, consecutive hospitalized patients with serologically confirmed acute PUUV infection. The collection of clinical data including routine laboratory measurements has been described in detail elsewhere [13].

All patients came from the Pirkanmaa area and were hospitalized in Tampere University Hospital, Finland for the median time of six days (range 2–15) during the period from September 1997 to February 2009. The median age of the patients was 40 years (ranging from 15 to 74 years), and 119 were males (69%). The concomitant diseases of the study group were arterial hypertension (n = 12), dyslipidemia (n = 7), CAD (n = 5), bronchial asthma (n = 6), atrial fibrillation (n = 3) and rheumatoid arthritis (n = 3). There were also patients with celiac disease, inflammatory bowel disease, valvular heart disease or neurological disease (n = 2 for each).

Definition of AKI

AKI was defined according to Kidney Disease: Improving Global Outcomes (KDIGO) criteria as an increase in plasma creatinine $\geq$1.5 times baseline, which was presumed to have occurred within the prior seven days [31]. The upper limits of the reference values for plasma creatinine (women 90 μmol/l and men 100 μmol/l) were taken as baseline levels. Thus, plasma creatinine $\geq$135 μmol/l in women, and $\geq$150 μmol/l in men was defined as AKI.

Genotyping

DNA was extracted from whole blood using a commercially available kit (QIAGEN Inc., Hilden, Germany). The gene polymorphisms of eNOS G894T (Glu298Asp, rs1799983) and iNOS G2087A (Ser608Leu, rs2297518) were genotyped with TaqMan® SNP Genotyping Assay (Life Technologies Ltd, Carlsbad, CA, USA) under standard conditions using the ABI Prism 7900HT Sequence Detection System (Taqman, Applied Biosystems, Foster City, CA, USA). Reaction volume used was 5 μl and it was prepared with TaqMan Genotyping MasterMix (Life Technologies Ltd, Carlsbad, CA, USA). Amplification data were analyzed with SDS 2.2 software (Taqman, Applied Biosystems, Foster City, CA, USA). The distributions of all SNPs did not deviate from the Hardy–Weinberg equation. The genotyping was successful in 167 of 172 patients (97%) for eNOS and in 166 patients (96.5%) for iNOS.

Statistical analysis

The highest and lowest values of continuous variables measured during acute PUUV infection were designated as maximum or minimum values. In order to describe the data, medians and ranges were given for skewed continuous variables and numbers for categorical variables. The allele frequencies were calculated, and the patients were grouped into carriers (including both homozygotes and heterozygotes) and non-carriers of specific alleles. Differences in the clinical severity of PUUV infection between groups were tested using Mann-Whitney U-test or Kruskal-Wallis test for numerical data and χ^2-test or Fisher`s exact test for categorical data, as appropriate. All p-values were two-tailed, and the statistical significance was considered at 0.05. Statistical analyses were performed using IBM SPSS software version 21.

Results

Clinical and laboratory findings

The clinical and laboratory findings of the 172 patients with acute PUUV infection are shown in Table 1. All patients suffered from serologically verified [32] and clinically typical PUUV infection, and they were examined and hospitalized during the acute phase of illness. Three patients (2%) were in clinical shock on admission to the hospital. AKI was found in 96 (56%) patients. Seven patients (4%) needed hemodialysis treatment during hospitalization. All patients recovered.

Association of eNOS G894T (Glu298Asp, rs1799983) polymorphism with clinical and laboratory findings. The genotype distributions and allele frequencies of eNOS G894T and iNOS G2087A polymorphisms are presented in Table 2. The rare genotype of eNOS G894T gene polymorphism was associated with the severity of AKI. The non-carriers of the G-allele of this eNOS polymorphism (TT-homozygotes, n = 10) had 1.9 times greater maximum level of

Table 1. The clinical and laboratory findings in 172 patients with acute Puumala hantavirus infection.

Clinical or laboratory variable	Median	Range
Days from the onset of fever*	4	1–14
Length of hospital stay (days)	6	2–15
Systolic blood pressure, minimum (mmHg)	113	74–170
Diastolic blood pressure, minimum (mmHg)	70	40–100
Change in weight (kg)**	2.1	0–12.0
Hematocrit, maximum	0.44	0.33–0.60
Hematocrit, minimum	0.36	0.25–0.46
Platelet count, minimum (x 10^9/l)	62	3–238
Leukocyte count, maximum (x 10^9/l)	10	3.9–31.2
CRP, maximum (mg/ml)	75	11–269
Creatinine, maximum (μmol/l)	185	51–1499
Interleukin-6, maximum (pg/ml)	14.5	1.3–107

Abbreviation: CRP, C-reactive protein.

Normal values: CRP < 10 mg/ml, creatinine ≤ 100 μmol/l for males and ≤ 90 μmol/l for females, platelet count 150–360 x 10^9/l, leukocyte count 3.4–8.2 x 10^9/l, hematocrit 0.35–0.50 for males and 0.35–0.46 for females.

*Equals to the onset of illness before the first blood test was taken.

**Change in weight during hospital stay reflects the fluid accumulation in the body during the oliguric phase.

doi:10.1371/journal.pone.0142872.t001

Table 2. The genotype distributions and allele frequencies in 167 patients for eNOS G894T (rs1799983) and in 166 patients for iNOS G2087A (rs2297518) out of 172 patients with acute Puumala hantavirus infection*.

	Genotype %			Allele %	
Polymorphism	homozygote common	heterozygote	homozygote rare	common	rare
eNOS(rs1799983)	59 (GG)	35 (GT)	6 (TT)	76 (G)	24 (T)
iNOS(rs2297518)	65 (GG)	33 (GA)	2 (AA)	81 (G)	19 (A)

Abbreviations: eNOS = endothelial nitride oxide synthase, iNOS = inducible nitride oxide synthase.

*The genotyping was successful in 167 patients for eNOS and in 166 patients for iNOS.

doi:10.1371/journal.pone.0142872.t002

serum creatinine than the carriers of the common G-allele (median 326, range 102–1041 vs. median 175, range 51–1499 μmol/l; p = 0.018, respectively: Fig 1A). The TT-homozygotes had numerically highest maximum creatinine level of all eNOS G894T genotypes, followed by the GT-heterozygotes and the GG-homozygotes, (median concentrations 326, 196, and 166 μmol/l, respectively, p = 0.061). Three out seven (43%) patients that needed hemodialysis treatment were T-allele carriers, and one of them was a TT-homozygote.

The non-carriers of the G-allele had higher maximum blood hematocrit value than the G-allele carriers (median 0.49, range 0.39–0.59 vs. median 0.44, range 0.33–0.60; p = 0.025, respectively; Fig 1B). The non-carriers of the G-allele had also higher maximum blood leukocyte count during acute phase of PUUV infection than the carriers of the common G-allele (GT-heterozygotes and GG-homozygotes, median 12.9, range 8.1–26.8 vs. median 9.9, range 3.9–31.2 $*10^9$/l; p = 0.047; Fig 1C).

The length of hospital stay was longer in the non-carriers of the G-allele (TT-homozygotes) when compared with the G-allele carriers (median 8 days, range 3–14 days vs. median 6 days, range 2–15 days; p = 0.032). There were no statistically significant associations with the lowest and highest blood pressure, change in weight, lowest platelet count, maximum C-reactive protein (CRP), maximum plasma IL-6, or the need of hemodialysis treatment, and the polymorphism of eNOS G894T (S1 and S2 Files).

Association of iNOS G2087A (Ser608Leu, rs2297518) polymorphism with clinical and laboratory findings. Carriers of the rare A-allele of the iNOS G2087A gene (AA-homozygotes and GA-heterozygotes, n = 59) had lower minimum systolic blood pressure during PUUV infection when compared with the non-carriers (GG-homozygotes, median 110, range 74–170 vs. 116, range 86–162 mmHg, respectively; p = 0.019). Furthermore, the A-allele carriers had also lower minimum diastolic blood pressure level than the non-carriers A-allele (median 68, range 40–90 vs. 72, range 48–100 mmHg, respectively; p = 0.003).There were no significant associations with change in weight, the lowest platelet count, maximum CRP, maximum plasma IL-6, or the need of hemodialysis treatment, and the iNOS polymorphism studied here (S1 and S3 Files).

Discussion

In this study we found that the TT-genotype of eNOS G894T polymorphism was associated with the severity of PUUV infection. PUUV-infected patients with the TT-homozygous genotype were prone to more severe AKI and longer hospital stay than the GT-heterozygotes or GG-homozygotes. They had also higher maximum leukocyte count and hematocrit values measured in the acute phase of the infection when compared with the other genotypes. The G894T polymorphism of eNOS gene was not associated with the depth of thrombocytopenia, which is in concordance with our previous finding that the severity of AKI does not associate

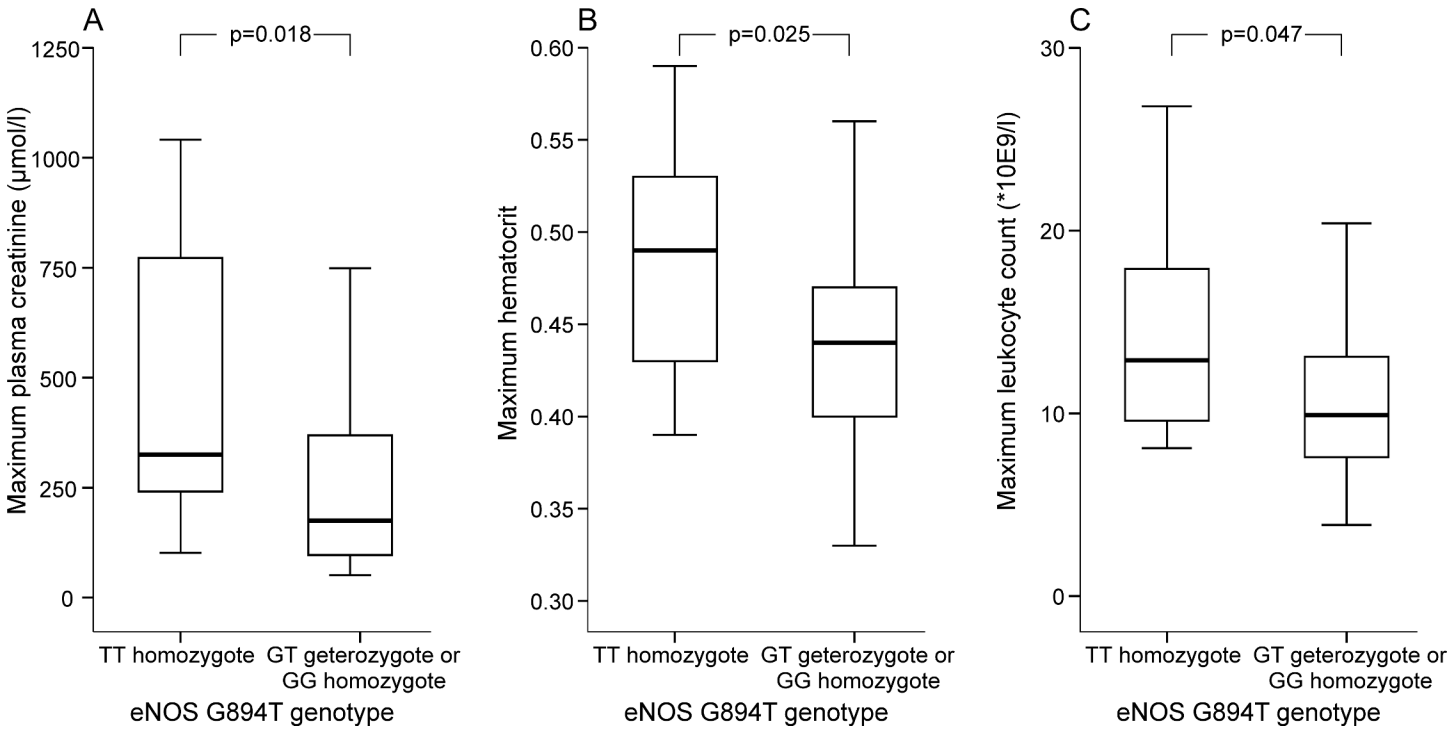

Fig 1. Box plots of the maximum plasma creatinine (A), hematocrit (B) and leukocyte count in TT-homozygotes (n = 10) and GT-heterozygotes or GG-homozygotes (n = 157) of eNOS G894T(rs1799983) polymorphism in 169 patients with PUUV infection. Box plot illustrates median (thick line inside box), 25th and 75th percentiles (box), and range (whiskers). Extremes and outliers have been omitted from the figure.

doi:10.1371/journal.pone.0142872.g001

with thrombocytopenia in acute PUUV infection in Finnish patients [33]. In some German studies severe thrombocytopenia has predicted severe AKI in PUUV infection [34, 35, 36]. We don't know the reason for these divergent results, but genetic factors in different populations may influence.

Furthermore, we also found that the iNOS G2087A gene polymorphism was associated with decreased blood pressure. The carriers of the rare A-allele of iNOS G2087A gene variant were the most susceptible ones to suffer from severe hypotension during the acute phase of infection. However, this iNOS polymorphism was not associated with the other clinical and laboratory markers reflecting disease severity.

NO is important in maintaining vascular homeostasis via relaxation of vascular smooth muscle cells and inhibition of growth, platelet activation and aggregation, as well as leukocyte adhesion to the endothelium [37]. NO is synthesized via a calcium-dependent process in endothelial cells from the amino acid L-arginine by the constitutively expressed eNOS, *i.e.* NOS3 [38]. The calcium-independent formation of NO via iNOS in macrophages is mainly expressed during inflammation and infection, and is triggered by cytokines [39]. In addition to maintaining vasodilatation of the vasculature and thus controlling blood pressure, eNOS has numerous vasoprotective and anti-atherosclerotic effects [40]. Polymorphism of the NOS3 gene, localized in 7q36 region of chromosome 7 [41], seems to have functional significance. The eNOS polymorphism G894T (Glu298Asp) results in a substitution of glutamate for aspartate at position 298 in eNOS exon 7, and this change has been associated with reduced basal NO production in the forearm of healthy subjects [42].

Increased capillary permeability and vascular leakage explain many clinical features of hantavirus infection, such as hemoconcentration, hypotension, shock and tissue edema.

Hantaviruses target the endothelial cells in the small vasculature [1, 9]. The data so far does not suggest that the infection would have direct cellular cytotoxicity to the endothelium, but virus-induced inflammation and host immune responses may contribute to the loss of endothelial barrier function [7, 43]. In this study, the rare G894T TT-homozygous genotype was associated with many of the clinical markers of severe PUUV infection, including hemoconcentration, leukocytosis, longer length of hospital stay, and especially the severity of AKI. As subjects with the common GG-genotype of eNOS G894T polymorphism had the mildest form of acute illness, the presence of this common G-allele might have some protective role during acute PUUV infection.

Effective renal blood flow is decreased in PUUV-induced AKI, but hypotension does not seem to explain the underlying intrarenal functional changes. Renal failure can occur without hypotension, and blood pressure levels do not correlate with the severity of AKI [9]. Renal tubular cells and mesangial cells produce NO, which is a significant regulator and also a protector of renal blood flow, glomerular filtration rate, and tubular function [44]. Interestingly, the increase in glomerular filtration rate and renal plasma flow in response to exogenous L-arginine infusion has been found to be blunted in subjects with the G894T allele of endothelial NOS, suggesting that this polymorphism is a functional variant also in human kidneys [45]. Thus, diminished NO bioavailability due to eNOS G894T polymorphism could predispose to the impairment of vascular and renal function through vasoconstriction.

Polymorphism of iNOS G2087A (Ser608Leu) leads to an amino acid substitution from serine to leucine in the coding region of exon 16 in NOS2 [30]. This gene variant is supposed to promote excessive NO formation and inflammation through increased iNOS activity within the A-allele carriers. In macrophages NO is a mediator of tumoricidal and bactericidal actions [30]. Previous studies have indicated that iNOS plays an important role in the origin of hypotension in septic shock. The A-allele carriage has been associated with increased susceptibility to septic shock [29]. Our finding indicated that the rare A-allele carriers (*i.e.* GA-heterozygotes and AA-homozygotes) of iNOS G2087A gene variant also suffered from more severe hypotension than the non-carriers of A-allele during acute PUUV infection.

We recently reported two cases of PUUV-HFRS with severe capillary leakage syndrome that were successfully treated with icatibant, a bradykinin B2-receptor antagonist [46, 47]. The activation of the kinin-kallikrein system and the subsequent formation of bradykinin is enhanced in hantavirus-infected endothelial cells [48]. The synthesis of eNOS is activated by bradykinin, which causes blood vessels to dilate via the release of NO and other endothelial autacoids [49]. Interestingly, it has been demonstrated that there is an association between HFRS and acute myocardial infarction and stroke in the acute phase of the disease, which may be partly explained by the increased platelet activation [50, 51]. Many studies have implicated eNOS polymorphism in the development of cardiovascular diseases [52], and the homozygous mutant (TT) genotype of G894T has conferred increased susceptibility to CAD [53, 54]. In the present study, the TT-homozygotes had the most severe AKI as evaluated by maximum creatinine levels, followed by the GT-heterozygotes and the GG-homozygotes. Although the number of the TT-homozygotes in our study here was only 10 patients, the results well correspond to the other findings above that have been associated with the G894T polymorphism of the eNOS gene. Taken together, our findings point to the possibility of impaired constitutive NO synthesis in the pathogenesis of acute hantavirus infection.

In conclusion, this study implies that eNOS G894T polymorphism may influence the clinical course of PUUV infection. This eNOS gene variant, associated with various vascular diseases, may also play some part in the endothelial and kidney dysfunction in the complex pathogenesis of acute PUUV infection. Among PUUV-infected patients, those with the rare TT-genotype of eNOS G894T polymorphism were more susceptible to severe AKI. Moreover,

patients with the rare A-allele of iNOS G2087A polymorphism had more severe hypotension during the acute phase of infection. To our knowledge this is the first study to associate eNOS and iNOS polymorphisms and disease severity of HFRS.

Supporting Information

S1 File. ENOS G894T polymorphism and associations with clinical and laboratory variables in PUUV infection.
(PDF)

S2 File. ENOS G894T polymorphism and associations with clinical and laboratory variables in PUUV infection.
(PDF)

S3 File. INOS G2087A polymorphism and associations with clinical and laboratory variables in PUUV infection.
(PDF)

Acknowledgments

The skillful technical assistance of Ms Katriina Ylinikkilä and Eini Eskola is greatly appreciated.

Author Contributions

Conceived and designed the experiments: SK OL SM TP ST HH PK IP JM. Performed the experiments: TP ST. Analyzed the data: SK HH SM OL JM. Contributed reagents/materials/analysis tools: TP ST PK SM JM. Wrote the paper: SK OL SM JM IP. Participated in revising the manuscript critically: SK OL SM ST TP IP JM. Produced the figure: IP HH. Read and approved the final manuscript: SK OL SM TP ST HH PK IP JM.

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