OUTI LAINE

Hemostasis and Complement Activation in Puumala Hantavirus Infection

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ACADEMIC DISSERTATION
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To all those who contribute
to hantavirus research

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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 Laine O, Mäkelä S, Mustonen J, Huhtala H, Szanto T, Vaheri A, Lassila R and Joutsi-Korhonen L (2010): Enhanced thrombin formation and fibrinolysis during acute Puumala hantavirus infection. Thromb Res 126: 154–8.

II Laine O, Mäkelä S, Mustonen J, Helminen M, Vaheri A, Lassila R and Joutsi-Korhonen L (2011): Platelet ligands and ADAMTS13 during Puumala hantavirus infection and associated thrombocytopenia. Blood Coagul Fibrinolysis 22: 468–72.

III Sane J, Laine O, Mäkelä S, Paakkala A, Jarva H, Mustonen J, Vapalahti O, Meri S and Vaheri A (2011): Complement activation in Puumala hantavirus infection correlates with disease severity. Ann Med, Apr 15, epub ahead of print.

IV Laine O, Joutsi-Korhonen L, Mäkelä S, Mikkelsson J, Pessi T, Tuomisto S, Huhtala H, Libraty D, Vaheri A, Karhunen P and Mustonen J (2012): Polymorphisms of PAI-1 and platelet GP Ia may associate with impairment of renal function and thrombocytopenia in Puumala hantavirus infection. Thromb Res 129: 611–5.

ABBREVIATIONS

A	activated
AG	antigen
ADAMTS13	a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13
ANDV	Andes virus
APTT	activated partial thromboplastin time
AT	antithrombin
C	complement component
CRP	C-reactive protein
DIC	disseminated intravascular coagulopathy
DOBV	Dobrava virus
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
F	(human coagulation) factor
FVIII:C	factor VIII activity
FDP	fibrin degradation product
F1+2	prothrombin fragments
GP	glycoprotein
HCPS	hantavirus cardiopulmonary syndrome
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPA	human platelet (allo)antigen
HTNV	Hantaan virus
IG	immunoglobulin
IL	interleukin
ISTH	the International Society of Thrombosis and Haemostasis

MAC	membrane attack complex
MP	microparticle
NE	nephropathia epidemica
NET	neutrophil extracellular trap
NP	nucleocapsid protein
NYV	New York virus
PC	protein C
PHV	Prospect Hill virus
PS	protein S free antigen
PT	prothrombin time
PUUV	Puumala hantavirus
RCO	ristocetin cofactor
RNA	ribonucleic acid
SAAV	Saaremaa virus
SANGV	Sangassou virus
SEOV	Seoul virus
SNV	Sin Nombre virus
TCC	terminal complement complex
TNF	tumor necrosis factor
TOPV	Topografov virus
TSP	thrombospondin
TT	thrombin time
TTP	thrombotic thrombocytopenic purpura
TULV	Tula virus
VWF	von Willebrand factor

ABSTRACT

Puumala hantavirus (PUUV) infection, also known as nephropathia epidemica (NE), is the most common cause of hemorrhagic fever with renal syndrome (HFRS) in Europe. The clinical illness is characterized by fever, headache, gastrointestinal symptoms and impaired renal function. Thrombocytopenia is encountered almost invariably, but bleeding manifestations usually remain mild. The clinical severity of acute PUUV infection varies from mostly mild to the rarely reported fatal cases.

To investigate markers of platelet and endothelial cell activation together with those of blood coagulation and fibrinolysis, 19 hospital-treated patients with serologically confirmed acute PUUV infection were included. Laboratory abnormalities were evaluated by the disseminated intravascular coagulation (DIC) scoring advocated by the International Society of Thrombosis and Haemostasis (ISTH). The major platelet ligands were all acutely altered, plasma fibrinogen and von Willebrand factor (VWF) being increased and plasma fibronectin decreased. ADAMTS13 activity was diminished. Prothrombin fragments F1+2 and D-dimer were elevated and all the natural anticoagulants antithrombin, protein C and protein S were decreased along with low platelet counts. Five patients (26%) fulfilled the ISTH criteria for DIC.

The activation of complement was studied by measuring the plasma levels of terminal complement complexes SC5b-9 and complement components C3 and C4 in 61 patients with acute PUUV infection. SC5b-9 and C3 levels were associated with several variables depicting the clinical severity of the disease. Patients with abnormal chest X-ray findings had higher levels of plasma SC5b-9 than those having normal images.

Several genetic polymorphisms related to platelet aggregation and activation, together with those of VWF and plasminogen activator inhibitor (PAI-1), were determined in 172 patients with acute PUUV infection. The A>G polymorphism of PAI-1 (rs2227631) was found to be associated with impairment of renal function. Patients carrying the functional T>C polymorphism of platelet glycoprotein (GP) VI (rs1613662) and the A>G polymorphism of VWF (rs1063856) had lower serum creatinine levels compared with non-carriers. The minor C-allele of GP Ia (human platelet alloantigen (HPA) -5) was associated with lower platelet count.

In conclusion, the findings imply several rearranged interactions between platelets and their ligands during acute PUUV infection. Enhanced thrombin formation and

fibrinolysis were observed, and thrombocytopenia was associated with decreased natural anticoagulants. In this series, these findings did not predict the clininical course of the disease. Complement activation via the alternative pathway was associated with the severe disease. Polymorphism of PAI-1, the major regulator of fibrinolysis, had an adverse impact on the outcome of kidney function in PUUV-induced HFRS.

TIIVISTELMÄ

Myyräkuumeen aiheuttaa hantavirusten ryhmään kuuluva Puumala-virus, joka on Euroopan yleisin munuaisoireisten verenvuotokuumeiden aiheuttaja. Taudinkuvalle tyypillisiä piirteitä ovat kuume, päänsärky, mahasuolikanavan oireet ja munuaisten toiminnan heikentyminen. Verihiutaleiden niukkuus todetaan lähes aina, mutta vuotooireet ovat yleensä lieviä. Puumala-viruksen aiheuttaman infektion vaikeus vaihtelee: infektio on yleensä lievä, mutta kuolemantapauksiakin esiintyy.

Tässä väitöskirjatyössä tutkittiin verihiutaleiden ja verisuonen seinämän aktivoitumiseen sekä veren hyytymiseen ja fibrinolyysiin liittyviä merkkiaineita 19:llä sairaalassa hoidetulla myyräkuumepotilaalla. Poikkeavat laboratoriotulokset pisteytettiin kansainvälisellä luokittelulla, jota käytetään konsumptiokoagulopatian (disseminoitunut intravaskulaarinen koagulopatia, DIK) diagnostiikassa. Muutoksia todettiin kaikkien tutkittujen verihiutaleisiin sitoutuvien molekyylien pitoisuuksissa. Fibrinogeenin plasmapitoisuus oli koholla, fibronektiinin alentunut ja plasman von Willebrand tekijän (VWF) aktiivisuus oli koholla. ADAMTS13-aktiivisuus todettiin alentuneeksi. Protrombiinifragmentit F1+2 ja D-dimeeri olivat kohonneet. Luonnollisten antikoagulanttien antitrombiinin ja proteiini C:n aktiivisuudet ja proteiini S:n vapaa antigeeni olivat alentuneet, ja alentumisella todettiin yhteys matalaan verihiutaletasoon. Konsumptiokoagulopatian kriteerit täyttyivät 5 potilaalla (26%).

Komplementtijärjestelmän aktivaatiota tutkittiin mittaamalla sen lopputuotteen SC5b-9:n ja osatekijöiden C3 ja C4 plasmapitoisuuksia 61 myyräkuumepotilaalla. Useiden kliinistä taudinkuvaa mittaavien muuttujien sekä SC5b-9- ja C3-tasojen välillä todettiin yhteys. Potilailla, joilla oli korkeat SC5b-9-tasot, oli myös enemmän poikkeavia keuhkokuvalöydöksiä.

Verihiutaleiden aggregaatioon ja aktivaatioon liittyviä geneettisiä polymorfioita samoin kuin VWF:n ja plasminogeenin aktivaattorin inhibiittori 1:n (PAI-1) polymorfioita tutkittiin 172 myyräkuumepotilaalla. PAI-1:n A>G (rs 2227631) polymorfiaan liittyi munuaisten toiminnan heikentyminen sairauden akuuttivaiheessa. Glykoproteiini (GP) VI:n toiminnallisen T>C (rs1613662) polymorfian ja VWF:n A>G (rs1063856) polymorfian kantajilla todettiin lievempi seerumin kreatiniinitason nousu myyräkuumeen akuuttivaiheessa kuin näitä polymorfioita kantamattomilla potilailla. GP Ia:n (human platelet alloantigen -5) harvinaiseen C-alleeliin liittyi sairauden akuuttivaiheessa matala verihiutaleiden taso.

Tulokset viittaavat verihiutaleiden ja niihin sitoutuvien molekyylien vuorovaikutukseen myyräkuumeen aikana. Trombiinin muodostus ja fibrinolyysi ovat lisääntyneet, ja verihiutaleiden niukkuus liittyy luonnollisten antikoagulanttien kulumiseen. Näillä merkkiaineilla ei voitu ennakoida taudin vaikeusastetta. Sen sijaan komplementtijärjestelmän aktivoituminen oikotien kautta liittyi vaikeaan taudinkuvaan. Fibrinolyysin säätelijän, PAI-1:n, polymorfialla oli epäsuotuisa vaikutus munuaisten toimintaan akuutissa myyräkuumeessa.

1 INTRODUCTION

Hantaviruses are the most widely distributed zoonotic viruses on the earth. In humans they cause two forms of severe disease, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) (Bi et al. 2008). Annually, HFRS affects approximately 200 000 people predominantly in Asia, with mortality rate between 6 to 15% in those with more severe form of disease (Muranyi et al. 2005). HCPS occurs much less frequently but with a considerably higher mortality rate. Hantaviruses are recognized by the World Health Organization as a significant public health problem (WHO 1983), and hantaviral diseases are included in the group of emerging infections (Schmaljohn et al. 1997).

As the disease burden caused to humans by hantaviruses is considerable, much effort has been invested in vaccine development, so far with limited success. There is still no specific therapy available for hantavirus infection, the treatment being symptomatic even in the most severe cases. The difficulties in developing a therapy against hantavirus diseases probably reflect the fact that the hantavirus pathogenesis is a complex multifactorial process which is not currently well understood. It is not known whether the therapeutics should target virus replication or the immune system and its virally induced reactions (Klingström et al. 2011).

Puumala hantavirus (PUUV) is the most common cause of HFRS in Europe, and 70% of reports come from Finland (Heyman et al. 2008). There is an increasing trend in incidence, and rates vary widely by season and region (Makary et al. 2010). The acute illness is characterized by high fever, headache, back and abdominal pains, visual disturbances, hemorrhages and acute impairment of renal function. Thrombocytopenia is almost invariably encountered in PUUV infection, but bleeding manifestations usually remain mild. The outcome of the disease is usually favorable in terms of long-term prognosis and case fatality (Vapalahti et al. 2003). However, 13 deaths due to PUUV infection were recorded in Finland during the period 1995-2008 (Makary et al. 2010).

To improve our understanding of the pathogenesis of hantavirus infection, a series of studies was undertaken in the acute phase of PUUV infection and at full recovery. The markers of platelet and endothelial cell activation as well as factors related to blood coagulation and fibrinolysis were determined. Several genetic polymorphisms related to platelet aggregation and activation as well as blood coagulation and fibrinolysis were

also investigated. The role of complement activation was further elucidated. As prevention and specific therapy of PUUV infection are yet to be discovered, predictive markers of disease severity are especially needed to identify the patients who need the most intensive symptomatic care as early as possible.

2 REVIEW OF THE LITERATURE

2.1 Puumala virus and other hantaviruses

2.1.1 Virology

Hantaviruses are enveloped negative-stranded ribonucleic acid (RNA) viruses with a diameter of 120 nm belonging to the genus *Hantavirus* within the family of *Bunyaviridiae*. The 12 kb-long RNA genome consists of three segments, the L (large), the M (medium), and the S (small) segments. The 6550 nucleotide-long L segment encodes an RNA-dependent RNA polymerase. The M segment is about 3680 long, and encodes a glycoprotein (GP) precursor cleaved into the G1 and the G2 surface glycoproteins. The S segment, about 1830 nucleotides long, encodes a nucleocapsid protein (NP) which encapsidates the genomic RNA (Plyusnin 2002). Hantaviruses replicate in the host cytoplasm and can infect many types of human cells, e.g. endothelial cells, macrophages and kidney glomerular cells *in vitro*. Pathogenic hantaviruses do not appear to cause cytopathogenic effects in infected cells. The virus is sensitive to heat (30 min 60°C), 70% ethanol and detergents (Vapalahti et al. 2003, Terajima et al. 2004).

The natural hosts for hantaviruses are persistently infected rodents, and each hantavirus species is associated with one (or a few closely related) specific rodent species. Hantavirus infection in the natural carrier is asymptomatic and long-lasting, and infected rodents continue to secrete the virus in urine, saliva and feces for months. Humans catch the virus by inhalation of aerosolized excreta and humans are believed to be dead-end hosts (Vapalahti et al. 2003, Klingström et al. 2011). However, the Andes virus (ANDV) has been reported to spread between humans (Martinez et al. 2005), and there is one report of transmission of PUUV via platelet transfusion (Sinisalo et al. 2010).

The earliest descriptions of hantavirus-caused disease in humans (HFRS) date back to Chinese writings as far back as 900 years ago. The Korean war in 1951–1953 introduced the illness to thousands of soldiers in the United Nations troops and HFRS became better known to the Western medicine. The disease caused by PUUV was first described by Swedish authors in 1934 (Myhrman 1934, Zetterholm 1934) and named nephropathia epidemica (NE). In Finland, the condition was extensively studied by Lähdevirta (1971), and the virus was first detected in samples collected near the vil-

lage of Puumala in 1977 (Brummer-Korvenkontio et al. 1980). Today, with over 40 hantavirus species known to exist (Heyman et al. 2009), hantaviruses are classified as emerging viruses and considered the most widely distributed zoonotic viruses on earth (Johnson 2001).

2.1.2 Geographic distribution

Hantaviruses comprise a genetically and antigenically diverse group of viruses present worldwide. They appear to have co-evolved with their hosts for millions of years and human infections are geographically restricted by the range of their primary hosts (Vapalahti et al. 2003). To demonstrate the worldwide presence of hantaviruses, selected species together with their rodent hosts, clinical disease and geographic distribution are presented in Table 1.

PUUV, along with its host, the bank vole, is found throughout Europe with the exception of Mediterranean coastal regions and most of the Iberian Peninsula and Greece. The large continuous forest areas in northern Europe probably contribute to the effective spread and general abundance of PUUV and NE. In temperate Europe forests are more fragmented which possibly explains the local patchy occurence of PUUV. The distributions of the Dobrava virus (DOBV) isolated from the yellow-necked mouse in Slovenia (Avsic-Zupanc et al. 1992, Avsic-Zupanc et al. 1995) and the Saaremaa virus (SAAV) from the striped field mouse in Estonia (Plyusnin et al. 1997b, Nemirov et al. 1999) overlap across most of Europe. An association of human disease with the Tula virus (TULV) or the Topografov virus (TOPV) has not been unequivocally shown. Amur virus has been identified in severe HFRS cases in the far eastern region of the Russia Federation (Yashina et al. 2001, Lokugamage et al. 2004). The Seoul virus (SEOV) has been distributed worldwide in rats travelling in ships (Lee et al. 1982).

The research prompted by HFRS during the Korean conflict (also known as Korean hemorrhagic fever) resulted in the identification of the Hantaan virus (HTNV) (Lee et al. 1978), the prototype member of the genus Hantavirus. HTNV is widely distributed in Asia, causing HFRS, the majority of cases being detected in China (Bi et al. 2008, Schönrich et al. 2008).

There is a large number of hantaviruses causing HCPS (formerly hantavirus pulmonary syndrome, HPS) throughout the Americas. First, the Sin Nombre virus (SNV) was detected in deer mice (*Peromyscus manipulatus)* in the southwestern United States (Nichol et al. 1993) and subsequently, the NY-1 virus (NYV) on an island off the coast of New York (Song et al. 1994) and ANDV in Argentina (Lopez et al. 1996). The Prospect Hill virus (PHV) is not known to associate with any human disease (Mackow et al. 2009).

There is a report of specific neutralizing antibodies against Sangassou hantavirus (SANGV) in serum samples of Guinean patients, and serosurveys suggest that hanta-

viruses cause human diseases at least in that part of Africa (Klempa et al. 2010). Hantavirus antibody-positive rodents have also been found across Australia. To date, there are no reports of human hantavirus disease on that continent (Bi et al. 2005).

Table 1. *Selected hantavirus species together with their rodent hosts, associated clinical diseases, and geographical distribution.*

Virus (abbreviation)	Rodent host	Disease	Distribution	Reference
Puumala (PUUV)	Bank vole *(Myodes glareolus)*	HFRS	Europe	Brummer- Korvenkontio et al. 1980
Dobrava (DOBV)	Yellow necked field mouse *(Apodemus flavicollis)*	HFRS	Europe	Avsic-Zupanc et al. 1992
Saaremaa (SAAV)	Striped field mouse *(Apodemus agrarius)*	HFRS	Europe	Nemirov et al. 1999
TULA (TULV)	European common vole *(Microtus arvalis)*	none	Europe	Plyusnin et al. 1994
Topografov (TOPV)	Siberian lemming *(Lemmus sibericus)*	none	Asia (Siberia), Northern Europe	Plyusnin et al. 1996
Amur	Korean field mouse *(Apodemus peninsulae)*	HFRS	Far East Russia	Yashina et al. 2001
Seoul (SEOV)	Rat *(Rattus rattus, rattus norvegicus)*	HFRS	worldwide	Lee et al. 1978
Hantaan (HNTV)	Striped field mouse *(Apodemus agrarius)*	HFRS	China, Korea, Russia	Lee et al. 1978
Sin Nombre (SNV)	Deer mouse *(Peromyscus maniculatus)*	HCPS	Western and Central United States, Mexico	Nichol et al. 1993, Elliott et al. 1994
New York (NYV)	White-footed mouse *(Peromyscus leucopus)*	HCPS	Eastern United States	Song et al. 1994
Andes (ANDV)	Long-tailed pygmy rice rat *(Oligoryzomys longicaudatus)*	HCPS	Argentina, Chile	Lopez et al. 1996
Prospect Hill (PHV)	Meadow vole *(Microtus pennsylvanicus)*	none	North America	Rowe et al. 1995
Sangassou (SANGV)	Allen's wood mouse (*Hylomyscus alleni)*	HFRS	Guinea, Africa	Klempa et al. 2006

HFRS=hemorrhagic fever with renal syndrome, HCPS=hantavirus cardiopulmonary syndrome.

2.1.3 Disease burden of hantavirus infections

Hantaviruses cause a wide range of human illnesses and present a significant public health threat. Globally 150 000–200 000 patients with HFRS are hospitalized every year. In the most endemic country, China, where the majority of HFRS cases are caused by HTNV, 40 000–60 000 cases have been reported annually in recent years. Over the period 1950 to 2001, the number of clinical cases reported in China was 1 400 000 and 45 000 deaths occurred (Bi et al. 2008). In more severe forms of HFRS, caused by DOBV and HTNV, the mortality rate is up to 10%. In HFRS with milder clinical course, as caused by PUUV and SEOV, the mortality rate is estimated to be between 0.1–1%. The incidence figure for HCPS, with approximately 200 cases reported annually in the Americas, is much smaller. However, a mortality rate of up to 40% and the record of human-to-human transmission of ANDV make it a considerable health problem (Vapalahti et al. 2003, Klingström et al. 2011).

PUUV is the most common cause of HFRS in Europe. The annual number of verified cases is about 2000 in Finland (incidence rate 31/100 000 population), 100–300 in Sweden and 100 in Norway (Bi et al. 2008). A recent Finnish study from the period 1995–2008 reported an increasing trend in incidence, and a wide variation in rates by season and region was noted (Makary et al. 2010). Of all the reported 22 681 cases in 1995–2008, 52% were hospitalized and 62% were males. Only 3% of all cases were registered as occupational disease, the majority of them related to farming and forestry (Makary et al. 2010).

2.2 Clinical hantaviral disease

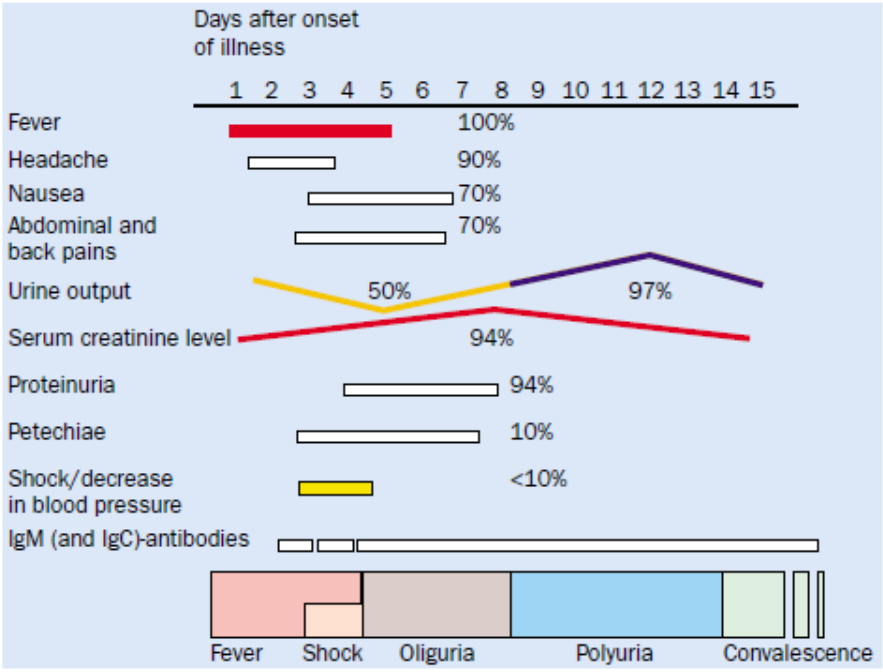
2.2.1 Clinical course of PUUV infection

The incubation period of PUUV is 2–4 weeks. The course of the disease may be divided into febrile, hypotensive, oliguric, diuretic and convalescent phases, but these phases overlap and may not be easy to recognize. The severity of the disease varies from subclinical to fatal, and the infection is thought to leave lifelong immunity (Vapalahti et al. 2003). Clinical PUUV infection in children seems rare, less than 5% of all reported patients with NE being under 15 years old. This may reflect the lesser severity of the disease in children (Settergren 1991, Mustonen et al. 1994b, Huttunen et al. 2011).

Clinical PUUV infection has a sudden onset with fever, headache and, after the second day, gastrointestinal symptoms such as nausea, vomiting and abdominal or back pain (Figure 1) (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010). Somnolence as well as ocular symptoms due to myopia are common (70%) in the early phase of the infection (Hautala et al. 2010). In the few severe cases hypotension and shock may develop rapidly. Renal symptoms begin around the

3rd or 4th day of the illness, typically manifested by transient proteinuria, microscopic hematuria and acute renal failure with oliguria followed by polyuria and spontaneous recovery. About 5% of all hospital-treated patients need transient hemodialysis treatment (Mustonen et al. 1994a, Braun et al. 2010).

Figure 1. *Schematic representation of typical clinical course of nephropathia epidemica. (Reprinted from Vapalahti et al. 2003. Copyright 2012, with permission from Elsevier.)*



As rare neurological manifestations, encephalitis, Guillain-Barre syndrome and acute disseminated encephalomyelitis have been reported (Mustonen et al. 1994a). Signs of inflammation (class IgM PUUV-specific antibodies, elevated protein level or leukocyte count) in the cerebrospinal fluid can, however, be detected in half of the hospitalized patients with acute PUUV infection (Hautala et al. 2010).

Almost all patients with acute PUUV infection recover with mortality of 0.08% (Makary et al. 2010). The long-term consequences of NE include most often mildly impaired renal function and elevated blood pressure (Mäkelä et al. 2000, Miettinen et al. 2006, Miettinen et al. 2009). Also chronic hormonal deficiencies and need of hormonal replacement therapy have been observed (Mäkelä et al. 2010). No association

between the severity of acute PUUV infection and the long-term outcome of patients has been noted (Miettinen et al. 2010).

2.2.2 Laboratory and radiological findings during PUUV infection

An immunoglobulin (Ig) M enzyme immunoassay (EIA) test based on recombinant full-length hantavirus NP is currently applied to confirm the diagnosis of acute hantavirus infection in humans (Vaheri et al. 2008). Immunographic IgM tests are also available for rapid diagnosis of several hantavirus infections (Hujakka et al. 2001, Hujakka et al. 2003). Previous hantavirus infection can be detected with IgG EIA (Kallio-Kokko et al. 1998). Though not in routine clinical use, polymerase chain reaction method can detect hantaviral RNA in blood (Plyusnin et al. 1977a), urine (Plyusnin et al. 1977a), saliva (Pettersson et al. 2008) and cerebrospinal fluid (Mähönen et al. 2007).

Thrombocytopenia is encountered in a majority of NE patients. The platelet count nadir usually occurs 4–5 days after the onset of fever, and normalizes most often within 7 days from the onset of initial symptoms (Lähdevirta 1971, Rasche et al. 2004, Braun et al. 2010). Table 2 presents data on platelet counts in Finnish (Lähdevirta 1971, Mustonen et al. 1994a), Swedish (Settergren et al. 1989, Sundberg et al. 2011) and German patients (Braun et al. 2010).

Table 2. *Platelet counts in patients with acute Puumala hantavirus infection.*

Study	Percentage of patients with thrombocytopenia	Additional data on platelet count (x 10^9/l)
Lähdevirta 1971 (n=34)	57%	20% with nadir < 100, 7 patients with nadir < 5
Mustonen et al. 1994a (n=126)	75%	mean 117, minimum 10, maximum 489
Settergren et al. 1989 (n=74)	52%	median 96, range 22–457, 15% with nadir < 50, 5% with nadir < 10
Braun et al. 2010 (n=75)	69%	mean 137±11, the lowest value observed 16
Sundberg et al. 2011 (n=106)	not available	median 95, range 17–404

A mildly elevated leukocyte count is frequently (36–57%) detected during the acute phase of NE (Lähdevirta 1971, Mustonen et al. 1994a, Braun et al. 2010). Anemia is observed in 50% of patients (Mustonen et al. 1994a), but also signs of hemoconcentra-

tion may be observed. Increased levels of plasma C-reactive protein (CRP) and serum creatinine concentration are found in almost all patients (96–100% and 96%, respectively) (Settergren et al. 1989, Braun et al. 2010).

Recent studies have discovered laboratory markers not yet in routine clinical use but predictive for the course of acute PUUV infection. A high plasma interleukin-6 (IL-6) concentration has been found to be associated with several variables reflecting severe disease (Outinen et al. 2010). High serum indoleamine 2,3-dioxygenase activity is linked to renal impairment (Outinen et al. 2011a), and a high plasma pentraxin-3 level predicts a low platelet count (Outinen et al. 2011b).

Abnormal cardiac signs may be detected by electrocardiogram or echocardiography in more than half of patients during acute PUUV infection (Mäkelä et al. 2009). The most common abnormal findings in the electrocardiogram are transient T-wave inversions, and in echocardiography impaired contractility of the left ventricle (Mäkelä et al. 2009).

Abnormal findings in chest radiography or computed tomography have been described in 16–53% of patients during the acute phase of NE (Lähdevirta 1971, Mustonen et al. 1994a, Linderholm et al. 1992, Kanerva et al. 1996, Paakkala et al. 2004). Pleural effusion, atelectasis and interstitial infiltrates are the most frequent observations (Paakkala et al. 2008). The changes are most often mild or moderate, being severe in only 2% of cases (Paakkala et al. 2008).

2.2.3 Hemorrhagic manifestations in hantavirus infections

In PUUV infection, bleeding manifestations usually remain mild. In a Finnish study petechiae (12%), epistaxis (11%), conjunctival bleeding (6%) and macroscopic hematuria (3%) were reported (Lähdevirta 1971). In a subsequent Swedish study 37% of patients had some hemorrhagic manifestation, most commonly epistaxis (21%), but conjunctival bleeding, metrorrhagia, macroscopic hematuria, melena, hematemesis and petechiae were also reported (Settergren et al. 1989). Mild gastrointestinal bleeding has been demonstrated by gastroscopy in nearly all NE patients (Nuutinen et al. 1992).

Hemorrhage of the pituitary gland has been demonstrated by MRI (Hautala et al. 2002, Hautala et al. 2010) and in autopsy (Valtonen et al. 1995, Hautala et al. 2002). Additionally, hemorrhage has been documented in the kidneys and other organs in fatal cases of NE (heart, liver, lungs and peritoneal cavity) (Valtonen et al. 1995).

Hemorrhagic manifestations of DOBV infection are more prominent than those of PUUV infection: thrombocytopenia is more severe and hemorrhagic complications are reported in 26–59% of patients (Avsic-Zupanc et al. 1999). SAAV infection is considered less severe than DOBV, but no comprehensive clinical comparisons are available (Vapalahti et al. 2003).

The worldwide-distributed SEOV causes an infection with milder thrombocytopenia and hemorrhagic symptoms and less severe clinical disease than HTNV (Kim et al. 1995, Zhang et al. 2011). Macroscopic bleeding symptoms are reported in 53% of patients, and typically severe bleeding symptoms are missing. Gingival hemorrhage (20%), melena (20%), macroscopic hematuria (16%) and epistaxis (16%) are common (Zhang et al. 2011).

HTNV causes an infection with a far more distinctive bleeding tendency than that typically found in other forms of HFRS. Macroscopic bleeding symptoms are found in nearly all patients (94%), and in particular severe hemorrhagic symptoms such as hematochezia (6%) and hematemesis or hemoptysis (5%) are present in this group of hantavirus infections (Zhang et al. 2011). In fatal cases the hemorrhagic triad of renal medulla, right atrial wall and anterior lobe of the pituitary gland has been observed (Lukes 1954).

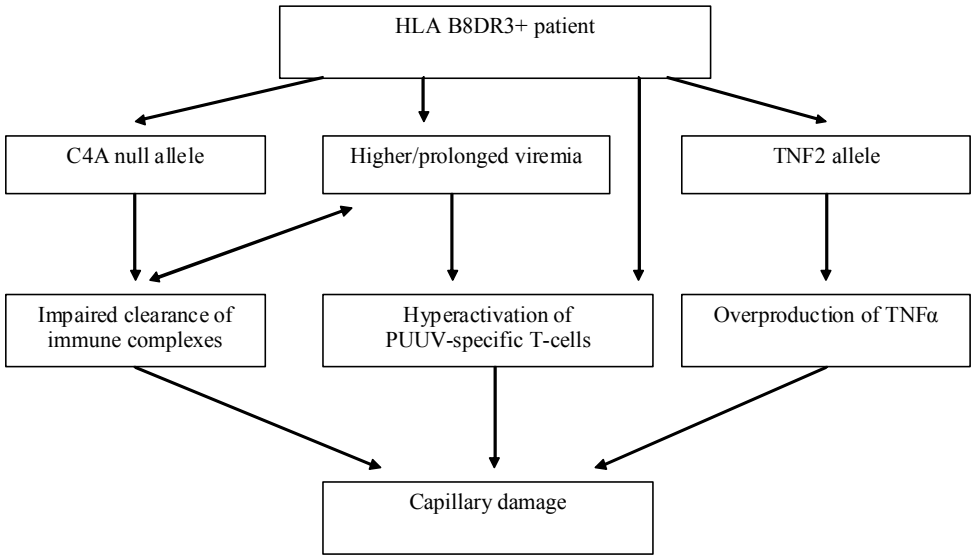
2.3 Hantaviruses and host genetic factors

Increased endothelial permeability and thrombocytopenia are the clinical hallmarks of hantavirus infection, but the pathogenetic mechanisms leading to them are not yet completely understood. The suggested pathophysiological processes include hyperactivation of PUUV-specific T-cells, overproduction of tumor necrosis factor (TNF) α and other cytokines, and platelet dysfunction (Mackow et al. 2009). With recognition of the genetic basis for individual variation in infectious diseases the role of certain genetic markers related to potentially important pathogenetic mechanisms has become the subject of intense studies in hantavirus as well as in other infections.

2.3.1 Human leukocyte antigen (HLA)-B8-DR3 haplotype

HLA are major cell surface antigens which present the antigen peptides to T cell. Increased amounts of CD8+ T cells have been detected in patients with acute hantavirus infection (Terajima et al. 2004). CD8+ T cells dominate in lymphocyte infiltrates of kidney biopsies taken in the acute phase of PUUV infection (Temonen et al. 1996) and their presence has been shown also in endobronchial biopsies and bronchoalveolar lavage fluid taken in the acute phase (Rasmuson et al. 2011). These findings may reflect the hyperactivation of PUUV-specific T-cells which could contribute to the endothelial damage by attacking the endothelial cells presenting PUUV epitopes (Terajima et al. 2004, Figure 2).

Figure 2. *Hypothesis of immunopathogenesis in PUUV-HFRS (modified from Terajima et al. 2004).*



HLA=human leukocyte antigen, TNF=tumor necrosis factor, PUUV=Puumala hantavirus.

Mustonen and coworkers (1996) studied the role of HLA B8 DR3 haplotype known to be associated with increased or altered immune response in the clinical course of acute PUUV infection. Individuals with the HLA B8, C4A*Q0 (C4 null allele), DRB1*0301, DQA1*0501 and DQB1*0201 alleles suffered from the most severe forms of the disease, which might be explained by impaired clearance of immune complexes due to the deletion of the C4A gene (Mustonen et al. 1996, Terajima et al. 2004). PCR positivity of blood or urine sediment specimens taken in the acute phase of PUUV infection has been found to be associated with the extended HLA B8 DR3 haplotype (Plyusnin et al. 1997a). This would imply that the levels of viremia are higher or the clearance of the virus is delayed in patients carrying the HLA B8 DR3 haplotype (Plyusnin et al. 1997a). The extended haplotype containing the HLA B8, DR3 and TNF 2 alleles is also accompanied by abnormal findings on chest radiography, especially pleural effusion (Paakkala et al. 2008), a finding reflecting capillary damage.

In regard to further associations between HLA haplotype and acute PUUV infection, the HLA B27 allele is known to be associated with a benign clinical course of the disease, reflected especially in a short treatment time in hospital (Mustonen et al. 1998a).

2.3.2 Cytokine gene polymorphisms

TNFα is known to induce vascular permeability and increase the expression of endothelial adhesion molecules, and intravenous injection of TNFα induces a number of signs and symptoms similar to those seen in acute hantavirus infection (Tracey and Cerami 1994). However, the association of elevated plasma TNFα levels with the clinical course of the hantavirus disease remains unclear (Mackow et al. 2009). There are also reports on elevated plasma levels of IL-1, IL-6 and IL-10 in HTNV (Krakauer et al. 1995) and PUUV (Linderholm et al. 1996) infections. However, only a very limited number of studies address the polymorphisms of the genes determining the amount of cytokine produced in patients with acute hantavirus infection.

Probably the most widely studied of the several polymorphisms of the promoter region of the TNFα gene on chromosome 6 is a polymorphic site at position -308, involving the substitution of a guanine (-308G) by an adenosine (-308A), the latter being associated with increased TNFα production (Wilson et al. 1997, Hajeer et al. 2001). The rarer TNF2 allele, associated with a high TNFα-producer phenotype, is more frequently found in patients with acute PUUV infection than in controls (Kanerva et al. 1998b). TNF2 allele carriers also suffer from more severe diasease compared with non-carriers as measured by several clinical and laboratory parameters (Mäkelä et al. 2001). However, these findings could be due to the strong linkage disequilibrium with the HLA B8 DR3 haplotype known to be associated with a more severe outcome of the disease (Mäkelä et al. 2002). In a Brazilian study TNF2 allele has also been suggested to represent a risk factor for developing HCPS (Borges et al. 2010).

The IL-1 gene family on chromosome 2, which codes for IL-1α, IL-1β and IL-1 receptor antagonist proteins, has been addressed in a study involving 87 patients with acute PUUV infection. None of the differences in the allele frequencies and genotypes of the genes in the IL-1 complex reached statistical significance (Mäkelä et al. 2001).

2.3.3 Platelet genetic properties

Decreased platelet counts and an increased bleeding tendency are almost inavariably present in acute hantavirus infection. Platelet genetic properties as well as polymorphisms of genes encoding factors involved in blood coagulation and fibrinolysis have an impact on the functional outcome of the hemostatic system. Platelet genetic properties, such as GP polymorphism, have been studied in a number of disease conditions, including diabetes mellitus, atherosclerosis and hepatitis C. Human platelet (allo)antigen (HPA) 1 and 2 polymorphisms have been found to be associated with the severity of dengue virus infection (Soundravally and Hoti 2007, Stephens 2010). Studies on the association of platelet genetic properties with the clinical disease in HFRS are, however, few.

β_3 integrin, an abundant surface receptor of both endothelial cells and platelets, is the cellular receptor for HTNV (Gavrilovskaya et al. 1999). β_3 integrins have a major role in platelet function and in maintaining capillary integrity, both known to be altered in acute hantavirus infection. Liu and coworkers (2008) addressed the role of β_3 integrin in regard to thrombocytopenia and disease severity in HTNV-induced HFRS. They measured the percentage of CD61 (β_3 integrin) -positive platelets and the intensity level of platelet CD61 by flow cytometry in 104 patients. The CD61 intensity in the oliguric phase correlated inversely with platelet count and positively with serum creatinine level and several other laboratory variables. According to the investigators, the intensity of platelet β_3 integrin may be indicative of disease severity in patients with HFRS (Liu et al. 2008).

Altered platelet activation and platelet-dependent hemorrhagic disorder are involved in the pathogenesis of hantavirus infection (Liu et al. 2008, Wu et al. 1992), and platelet activation and adhesion are mediated by platelet membrane GPs. Polymorphisms in the genes encoding for the most abundant platelet receptor, platelet GP IIb/IIIa or integrin $\alpha IIb\beta_3$ (HPA-1 and HPA-3), have been linked to increased platelet adhesiveness and aggregation in some studies (Bray 1999, Reiner et al. 2000). The functions of β_3 integrin in platelet adhesion and activation, maintenance of capillary integrity and cellular entry of pathogenic hantaviruses prompted a study by Liu and coworkers (2009a) focusing on the association of HPA-1 and HPA-3 polymorphisms of GP IIbIIIa in patients with HTNV-HFRS. The study cohort comprised 104 patients, and 100 healthy unrelated blood donors served as controls. The investigators found no association between HPA-1 polymorphism, which is present on GP IIIa (β_3), and the disease or its severity. On the other hand, there were significant differences in the HPA-3 polymorphism both between HFRS patients and controls and between HFRS patients of different clinical types. Based on these findings, the investigators considered HPA-3 polymorphism to constitute one possible inherited risk factor associated with susceptibility to hantavirus infection and disease severity in HFRS (Liu et al. 2009a).

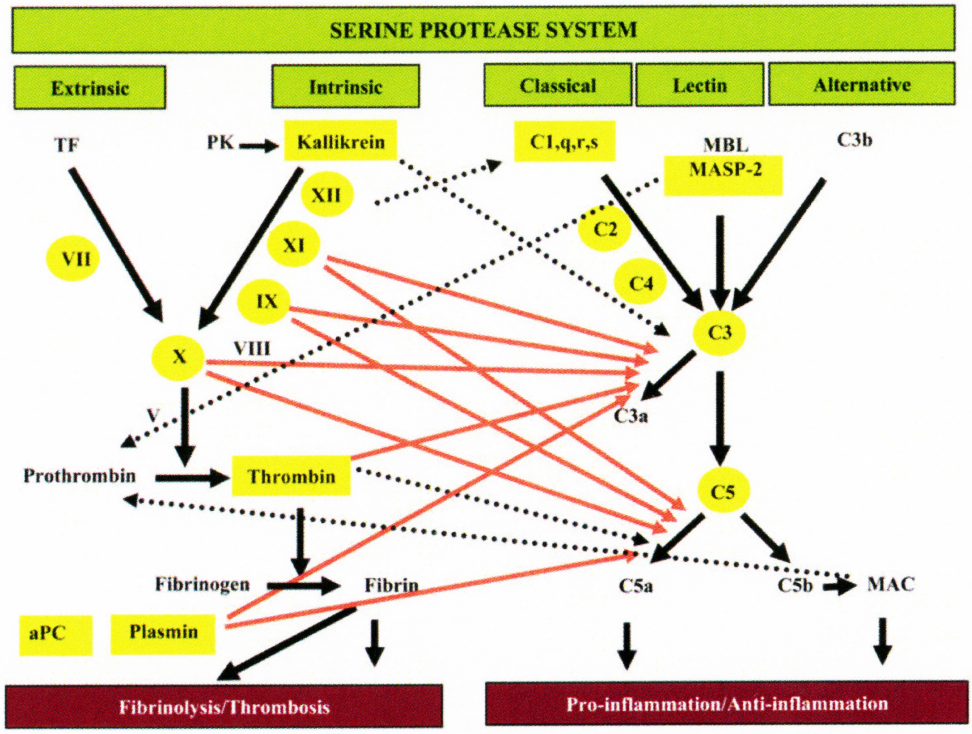
2.4 Role of complement activation in hantavirus infection

2.4.1 Molecular mechanisms between the complement and coagulation systems

The complement system is a proteolytic cascade composed of serine proteases with three major pathways of activation, the classical, the alternative and the lectin-dependent. These differently activated pathways all converge on complement component C3, which has a key function in the complement system (Walport 2001). When the complexes are formed in the absence of a target membrane in the fluid phase, C5b-9 binds to S-protein (vitronectin), forming a non-lytic soluble SC5b-9 terminal complement complex (TCC). The cytolytic membrane attack complex (MAC) is the final product

of the complement cascade (Podack et al. 1979). The complement and coagulation systems are commonly described as separate cascades, even though both belong to a complex inflammatory network (Rittirsch et al. 2008) and exhibit some similar characteristics in respect of the specialized functions of their activators and inhibitors (Figure 3).

Figure 3. *Simplified model of the serine protease system depicting the complex interplay between the coagulation/fibrinolysis cascade and the complement system. The serine proteases of the complement, coagulation and fibrinolysis systems are highlighted in yellow. The black dotted arrow bars show previously known interactions between these systems. The red arrows identify the new paths of complement activation by the coagulation/fibrinolysis factors, resulting in the generation of C3a, and C5a. aPC=activated protein C, MAC=membrane attack complex, MBL=mannan-binding lectin, PK=prekallikrein. (Amara U et al. 2010. Copyright 2010. The American Association of Immunologists, Inc.)*



Multiple links have been found between the complement and coagulation cascades. Activated (a) human coagulation factor (F) XII is known to be able to activate complement factor C1r and thereby initiate activation of the classical pathway, and the C1r esterase inhibitor suppresses both complement activation and intrinsic coagulation cascade (FXIIa, kallikrein; Ghebrehiwet et al. 1981). Thrombin is capable of generating the complement activation product C5a in the absence of C3 (Huber-Lang et al. 2006) and possibly even in the absence of C4 (Clark et al. 2008). C5a is able to induce tissue factor (TF) activity and may thus be involved in the activation of the extrinsic coagulation pathway (Ikeda et al. 1997). Importantly, the terminal complement complex (TCC; SC5b-9) is able to catalyze prothrombin cleavage to thrombin and thus increase platelet prothrombinase activity (Wiedemer et al. 1986). On the other hand, C5a is also known to have fibrinolytic activity (Wojta et al. 2002). The lectin pathway of the complement system and coagulation cascade are linked by mannan-binding lectin –associated serine protease 2, which has been found to promote fibrinogen turnover by cleaving prothrombin into thrombin (Krarup et al. 2007).

Amara and coworkers (2010) described several molecular mechanisms linking the coagulation and fibrinolysis cascades with the central complement components C3 and C5 (Figure 3). Thrombin, FXIa, FXa, FIXa and plasmin were all found to cleave C3 and C5 into products displaying molecular weights identical to the native anaphylatoxins C3a and C5a. Cleavage products exhibited chemoattraction of human mast cells and neutrophils. FXa-induced cleavage of C3 was suppressed by fondaparinux and enoxaparin, the selective FXa inhibitors. The investigators also reported a very early appearance and correlation of coagulation (thrombin-antithrombin complexes) and the complement activation product C5a in plasma samples from 12 patients with multiple injuries (Amara et al. 2010). This study further stresses the extensive cross-talk between molecular mechanisms active in blood coagulation and fibrinolysis with the complement system.

2.4.2 Complement activation and clinical hantaviral disease

Studies addressing the possible association between complement activation and clinical outcome of hantavirus disease are few. Wang and coworkers (1986) observed the peak of complement activation in HTNV-HFRS to appear during the hypotensive phase and gradually disappear in the convalescent phase. A study by Lee reported the appearance of circulating immune complexes along with decreased C3 and normal C4 levels in the serum of patients with HFRS induced presumably by HTNV. A significant decrease of serum C3 was most evident in patients with disseminated intravascular coagulation (DIC; by Colman's criteria) (Lee 1987).

The complement system is also known to be activated in PUUV-HFRS. A study by Paakkala and coworkers (2000) included 25 hospital-treated patients with serologically

confirmed acute PUUV infection. The complement system was activated in 23 of them, an activation through the alternative route being a more frequent finding. However, classical pathway activation was associated with more severe clinical course of the disease (Paakkala et al. 2000).

2.5 Hemostatic system and hantavirus infection

2.5.1 Platelets, their functions and mechanisms of thrombocytopenia

Platelets are small anucleate cells which are derived from megacaryocytes in the bone marrow. There are approximately one trillion platelets circulating in the blood of an adult human (platelet count reference range 150–360 x 10^9/l), and their average daily consumption ranges between 7–10 x 10^9/l. If not consumed earlier, they are destroyed in the spleen after 8–10 days.

The primary physiological role of the platelets is to initiate blood clotting to block circulatory leakage in the damaged vessel endothelium (Italiano and Hartwig 2007, Semple et al. 2011). As a functional response to vessel wall injury platelets manifest adhesion, activation-secretion and aggregation. The adhesion of platelets is mediated by specific membrane receptors, platelet GPs, known to be polymorphic (Rozman 2002).

Platelets also influence the innate and adaptive immune responses. They can synthesize and store molecules in the alpha and dense granules and lysosomes (Semple et al. 2011). Following activation with thrombin, platelets are capable of secreting over 300 different proteins, and some of them are clearly involved in processes other than blood clotting (Coppinger 2007, Semple et al. 2011). Platelet-derived CD154 supports B cell differentiation and immunoglobulin class-switching, thus promoting adaptive immune responses (von Hundelshausen and Weber 2007). Platelet-expressed CD154 also enhances leukocyte recruitment to inflammatory sites by inducing upregulation of intercellular and vascular adhesion molecules (Andre et al. 2002), and also augments $CD8^+$ T cell responses (Elzey et al. 2008). Other examples of the ways platelets promote innate immunity include expression of toll-like receptors (Shiraki et al. 2004) and ligands for triggering receptors expressed on myeloid cells 1 (Haselmayer et al. 2007). Platelets have been shown to have roles in the initiation of inflammation, angiogenesis, atherosclerosis, lymphatic development and tumor growth (Smyth et al. 2009).

The most common mechanism of thrombocytopenia is accelerated platelet destruction caused by immunologic or nonimmunologic processes. Thrombocytopenia may also result from deficient platelet production as a consequence of hypoplastic megacaryocytes, ineffective thrombopoiesis, disorders in thrombopoietic control or hereditary thrombocytopenia. Abnormal pooling of the platelet mass may produce thrombocytopenia, as seen in the various disorders associated with splenomegaly (Rodgers

2009). In viral infections, the development of thrombocytopenia often involves multiple mechanisms. In dengue virus infection, bone marrow suppression, enhanced immune response and activation of endothelial cells all contribute to a decrease in platelet count (Srichaikul and Nimmannitya 2000, Sosothikul et al. 2007). The mechanisms of thrombocytopenia in chronic hepatitis C infection include bone marrow suppression and platelet antibody formation (Olariu et al. 2010). In human immunodeficiency virus (HIV) infection, thrombocytopenia is generally associated with decreased platelet survival (Savona et al. 1985). In addition, defective megacaryopoiesis and splenomegaly may contribute to thrombocytopenia particularly in patients with advanced disease (Savona et al. 1985, Zauli et al. 1992, Kunzi et al. 1993).

2.5.2 Platelets in hantavirus infection

HTNV-induced HFRS is known for severe hemorrhagic problems. Potential causes of thrombocytopenia during HFRS as cited in the literature involve immune mechanisms, vascular injury and DIC (Cosgriff 1991, Cosgriff and Lewis 1991). Immune complexes on platelet surfaces in HFRS patients are thought to induce platelet aggregation, as well as lysis of platelets by complement and phagocytosis of platelets by mononuclear phagocytes. Vascular injury contributes to decreased platelet counts through platelet adherence and aggregation to subendothelial connective tissue. Platelet dysfunction (Cosgriff et al. 1991) and the "exhausted platelet syndrome" (the continued circulation of platelets which have already been activated) have some role in bleeding tendency, as well as uremia and inhibition of platelet aggregation by fibrinogen degradation products (Cosgriff and Lewis 1991).

In a study of coagulation, fibrinolysis, kinine and immune systems in HTNV-induced HFRS, Lee (1987) showed that the most frequently observed abnormality in the coagulation profile was a decrease in the platelet count. The life-span of platelets was significantly shortened, and the peripheral blood smear revealed giant platelets. In vitro aggregations to ADP, epinephrine and collagen were decreased (Lee 1987). The finding of decreased platelet aggregation and release in HTNV-HFRS were later verified by Xiang and coworkers (1990). In the bone marrow an increase in the number and size of megakaryocytes and active formation of platelets from the megakaryocytes was noted in an electron microscopic study (Lee 1987). Bone marrow aspiration has also been performed in five cases of PUUV-HFRS by Lähdevirta (1971), and plasma cell reaction together with increased megakaryocyte formation and number of immature megakaryocytes were noted.

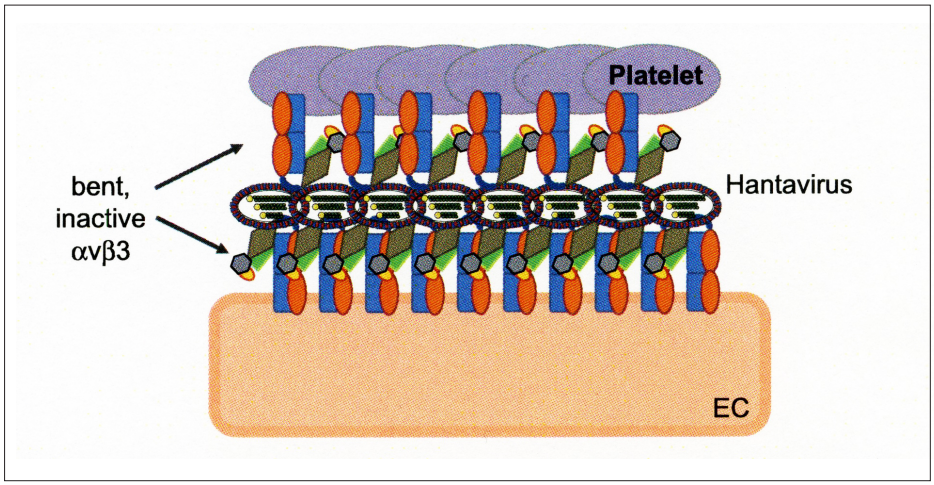
Decreased platelet counts and bleeding of variable severity occur in all hantavirus infections. However, it is not yet clarified why certain hantavirus species cause deeper thrombocytopenia and more severe hemorrhagic problems than others. The

divergence of hantavirus surface GPs (G1 and G2) is likely to contribute to pathogenic reponses to individual viruses (Mackow and Gavrilovskaya 2001).

2.5.2.1 β_3 integrin

Gavrilovskaya and coworkers (1999) published findings demonstrating that the cellular entry of pathogenic hantaviruses (HTNV, SEOV and PUUV) but not the nonpathogenic PHV is mediated by β_3 integrins, which are abundantly present on the surfaces of platelets, endothelial cells and macrophages. The pathogenic hantaviruses bind the plexin-semaphorin-integrin domains present at the apex of inactive, bent $\alpha_v\beta_3$ integrins (Raymond et al. 2005). The entry of pathogenic hantaviruses to endothelial cells could be inhibited by antibodies to $\alpha_v\beta_3$ integrins and the integrin ligand vitronectin. The cellular entry of the nonpathogenic PHV was blocked by $\alpha_5\beta_1$-specific sera and the integrin ligand fibronectin. Interestingly, a mouse–human hybrid β_3 integrin-specific Fab fragment c7E3 (ReoPro) inhibited the infectivity of HTNV, SEOV, PUUV, as well as SNV and NY-1 associated with HPS (Gavrilovskaya et al. 1999). As β_3 integrins regulate platelet function and vascular permeability, the pathogenic hantavirus regulation of β_3 integrin possibly impacts platelet functions and contributes to the thrombocytopenia and loss of vascular integrity seen in hantavirus infection (Mackow et al. 2009).

Figure 4. *Model depicting cell-associated hantavirus directing the adherence of quiescent platelets to surface of infected endothelial cells. (Mackow et al. 2009. Copyright permission no 302/11/2011 by Schattauer GmbH.)*



EC=endothelial cell.

The interaction between pathogenic hantaviruses, platelets and endothelial cells was further discussed by Gavrilovskaya and coworkers (2010) based on observations of pathogenic hantaviruses binding quiescent platelets and platelets binding to endothelial cells infected with pathogenic hantaviruses. They concluded that a platelet layer covers the surface of infected endothelial cells (Figure 4) which changes the appearance of these cells. This may alter cellular immune responses, platelet activation and endothelial cell functions and result in a loss of vascular integrity. These could be mechanisms by which hantaviruses cause thrombocytopenia and induce hypoxia (Gavrilovskaya et al. 2010).

2.5.2.2 Platelet ligands and serum thrombospondin-1

Vitronectin, a ligand of platelet $\alpha IIb\beta_3$ integrin, is produced mainly by the liver and stored in platelet alpha-granules. Vitronectin plays a role in platelet adhesion, aggregation and fibrinolysis (Seiffert et al. 1996, Reheman et al. 2005). Vitronectin is also involved in complement activation (Podack et al. 1979). Vitronectin gene expression is up-regulated in inflammation and has been identified as an acute-phase reactant (Korc-Grodzicki et al. 1988). Liu and coworkers (2009b), however, found decreased serum vitronectin levels in 112 patients with HTNV-HFRS from China, and the lowest levels of vitronectin were measured during the hypotensive and oliguric phases. There was no association between serum vitronectin levels and the clinical severity of the disease. The authors consider the decreased alteration of vitronectin as a possible consequence of inflammation, abnormal coagulation and liver damage in HFRS (Liu et al. 2009b).

Another ligand of β_3 integrin, fibronectin, is derived mainly from vascular endothelial, fibroblast and liver cells. Fibronectin is firmly bound to fibrinogen and fibrin at low temperatures (0–4°C) *in vitro* and subsequently released as the temperature rises (Ruoslahti and Vaheri 1975). Fibronectin participates in plug formation by gluing platelets to each other especially under arterial share rates (Ni et al. 2003), and it is thought to play a role in the infectious disease process due to its participation in adhesion and signal transduction pathways (Orem et al. 2002, Ruiz Martin et al. 2004). Serum fibronectin concentrations have been found to be increased and associated with disease severity in 112 patients with HTNV-HFRS (Han et al. 2010). However, the temperature at which the ELISA analyses were carried out was not provided.

Thrombospondin (TSP)-1 is a matricellular GP released mainly from the alpha-granules of activated platelets. TSP-1 plays a role in several cell-matrix or cell-cell interactions, which influence platelet function, angiogenesis, tumor biology, wound healing and vascular disease (Taraboletti et al. 1990, DiPietro et al. 1996, Frangiogannis et al. 2005). The plasma level of TSP-1 is usually low in the healthy vascular system and overexpressed during inflammation or injury (Esemuede et al. 2004, McMaken et al. 2011).

The serum TSP-1 levels of the above mentioned 112 Chinese HTNV-HFRS patients were found to be significantly decreased in the febrile and hypotensive phases compared with the controls (30 healthy volunteers without a history of HFRS-like disease) (Liu et al. 2008b). The reduction in TSP-1 level was more profound in patients with severe clinical disease. The investigators assumed that the interaction of hantavirus and $\alpha IIb\beta_3$ integrin may lead to the activation of platelets and release of the contents of alpha-granules. In the course of the HFRS platelets become more seriously affected and in the severe cases thrombocytopenia emerges, resulting in decreased production and release as well as increased consumption of TSP-1 (Liu et al. 2008b).

2.5.3 Activation of coagulation and fibrinolysis

Vascular injury is thought to play a major role in the activation of the coagulation system in HFRS. Exposure of subendothelial tissue leads to activation of FXII, which results in the generation of thrombin via the intrinsic pathway. Exposure of tissue factor on the surface of endothelial cells and platelets, again, can activate extrinsic coagulation pathway (Cosgriff and Lewis 1991). Tissue factor may also be produced as a response to IL-1 and TNF-α (Bevilacqua et al. 1986). Tissue factor might possibly be elaborated by monocytes during the course of HFRS (Edwards and Rickles 1984), but so far there are no published data on this mechanism in hantavirus infection.

In a study of coagulopathy in patients with HTNV-HFRS Lee (1987) noted a marked prolongation of the bleeding time along with a decrease in platelet counts. Prothrombin time (PT) and partial thromboplastin time were prolonged in 30–80% of patients and normalized within 2 weeks from the beginning of the disease. The activities of coagulation factors II, V, VIII, IX and X were decreased, but factor VIII -related antigen was increased and Von Willebrand factor ristocetin cofactor (VWF:RCo) activity remained normal. The blood fibrinogen concentration was decreased in 20% of patients and the fibrinogen half-life was markedly shortened. Thrombin time (TT) was prolonged and increased levels of fibrin degradation products (FDP) were noted. Lowered plasma levels and activities of plasminogen, alpha 2 -plasmin inhibitor and antithrombin (AT) were observed. Thromboelastogram confirmed the presence of procoagulant activity (Lee 1987).

The findings of Xiang and coworkers (1990) in 134 patients with HTNV-HFRS are consistent with those of Lee in 1987. Both TT and PT were prolonged. The concentrations of plasma fibrinogen and plasminogen and the activity of AT were all decreased (Xiang et al. 1990).

Settergren and coworkers (1989) observed prolongation of activated partial thromboplastin time (APTT) and/or PT in 41% of patients with PUUV infection. Elevated blood levels of FDP were also noted in some cases (Settergren et al. 1989). Previously,

Lähdevirta (1971) had observed consistent abnormalities in coagulation tests in a small number of patients included in his larger study of PUUV-HFRS.

2.5.4 Disseminated intravascular coagulopathy

In the literature DIC is considered to be common in HFRS induced by HTNV (Cosgriff and Lewis 1991). There are also published data supporting a relationship between laboratory findings of DIC, risk of hemorrhage and overall disease severity in patients with HFRS (Luo et al. 1979). However, the diagnostic criteria for DIC vary and the scoring system recommended by the International Society of Thrombosis and Haemostasis (ISTH) (Taylor et al. 2001) is not applied.

In a study including patients admitted to Seoul National University Hospital and the Army General Hospital during the period 1979–1986, Lee (1987) observed DIC by Colman's criteria in 100% of patients on the 4th day of clinical illness. Decreased platelet count, prolonged bleeding time together with PT and PTT, decreased levels of coagulation factors, activation of the fibrinolytic system, presence of plasma procoagulant activity and fibrin thrombi in autopsy cases were also demonstrated (Lee 1987).

Settergren and coworkers (1983) described a patient with NE and DIC from Sweden. The symptoms and laboratory findings included macroscopic hematuria, increased levels of FDP, decreased prothrombin complex and prolonged APTT. In 1995 four fatal cases of NE and presumed DIC from Finland were presented. All patients had macroscopic hematuria and signs of bleeding in autopsy, but except for low platelet counts no data on markers of coagulation and fibrinolysis were presented (Valtonen et al. 1995).

In a study of 74 patients with NE Settergren and coworkers (1989) found that 5% of them developed DIC, which was defined as clinically evident bleeding, positive ethanol gelation test, increased serum FDP levels and platelet count below 100 x 10^9/l.

A large outbreak of PUUV-induced HFRS occurred in northern Sweden in 2006–2008, with several patients falling critically ill with thromboembolic complications and life-threatening bleeding. Sundberg and coworkers (2011) included 106 patients in their study designed to establish the variability of DIC in consecutive patients and to evaluate different DIC scores as prognostic markers for severe illness. The investigators based their diagnosis of DIC on the scoring system recommended by the ISTH (Taylor et al. 2001) (Table 3), which they modified by including correction for the fibrinogen/CRP ratio. They found that 28% of the patients met the criteria for overt DIC, and the diagnosis of DIC correlated with more severe disease. ISTH score with fibrinogen/CRP ratio was predictive for moderate or severe illness and bleeding of moderate or major importance (Sundberg et al. 2011).

Table 3. *The ISTH score for overt DIC. A score of 5 points or more is indicative of DIC (modified from Taylor et al. 2001).*

Test	Result	Points
Platelet count (x10^9/l)	<100	1
	<50	2
D-dimer (mg/l)	>0.5	2
	>4.0	3
Thromboplastin time (%)	<70	1
	<40	2
Fibronogen (g/l)	<1.0	1

ISTH=the International Society of Thrombosis and Haemostasis, DIC=disseminated intravascular coagulopathy.

3 AIMS OF THE STUDY

The aims of the present study were to assess

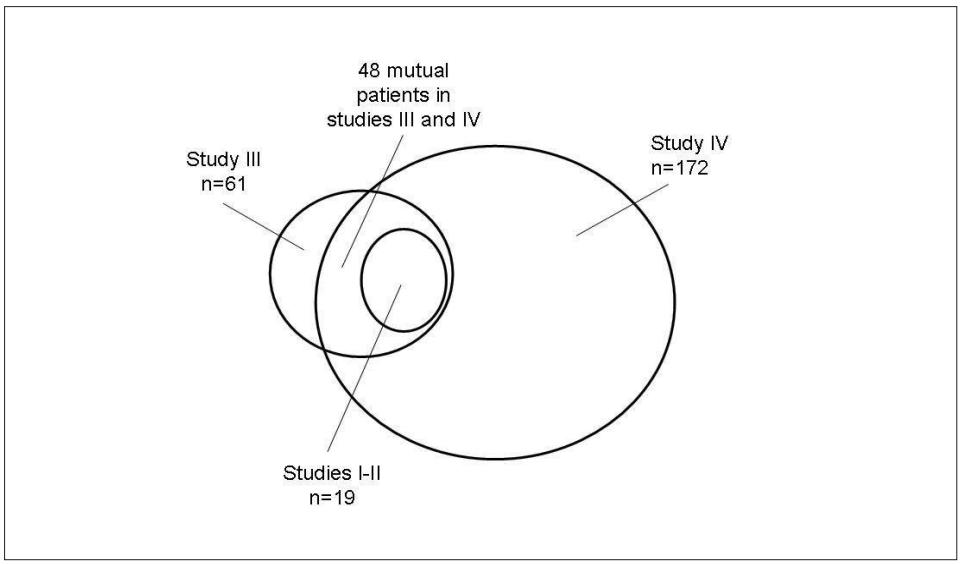
1. the activation of coagulation and fibrinolysis in acute PUUV infection and relate the findings to the clinical course of the disease
2. whether disseminated intravascular coagulopathy could be diagnosed with the ISTH score during acute PUUV infection
3. the adhesive platelet ligands related to endothelial function and acute phase reactions in PUUV infection and relate the findings to the clinical course of the disease
4. the complement activation along with the clinical disease in PUUV infection
5. the possible associations between genetic polymorphisms involved in platelet function, blood coagulation and fibrinolysis and the clinical course of PUUV infection

4 PATIENTS AND METHODS

4.1 Patients

All patients came from the Pirkanmaa region of Finland and were hospitalized in Tampere University Hospital with serologically confirmed acute PUUV infection. Studies I–III were carried out in Tampere University Hospital, the University of Tampere School of Medicine, the University of Helsinki Haartman Institute and the Laboratory of Helsinki University Central Hospital. Study IV was carried out in Tampere University Hospital and the University of Tampere School of Medicine. As depicted in Figure 5, all patients in studies I–II (n=19) were included in study III (n=61) and study IV (n=172). Studies III and IV had 48 patients in common.

Figure 5. *Distribution of patients participating in studies I–IV.*



Studies I–II involved 19 patients (17 men, median age 38 years, ranging from 30–64 years) selected from those who participated in a larger prospective study during the period from September 2000 to December 2002. Selection was based on the availability of laboratory samples.

Regarding the medical history of the patients in studies I–II, there were two patients with a neurological disease (multiple sclerosis and epilepsy) and two patients with dyslipidemia. One patient had coronary heart disease, one arterial hypertension and one paroxysmal atrial fibrillation. One patient with chronic inflammatory bowel disease was treated with mesalazine medication. Two patients used anti-platelet therapy (aspirin). No patient was receiving anticoagulation or immunosuppression.

Study III was prospective involving 61 patients (44 men, median age 46 years, ranging from 22–77 years) and conducted between September 2000 and January 2004. The study group included patients with multiple sclerosis, epilepsy, Sjögren's syndrome, fibromyalgia, hypothyroidism, paroxysmal atrial fibrillation, Crohn's disease and sarcoidosis, one each. Two patients had dyslipidemia, coronary heart disease, asthma or prostatic hyperplasia. There were eight patients with arterial hypertension.

Study IV, involving 172 prospectively collected patients (118 men, median age 40 years, ranging from 15 to 74 years) was conducted during the period from September 1997 to February 2009. Patients' medical histories included arterial hypertension (12 patients), dyslipidemia (7 patients), asthma (6 patients), coronary heart disease (5 patients), paroxysmal atrial fibrillation and rheumatoid disease (3 patients each). Two patients had celiac disease, inflammatory bowel disease, valvular heart disease or a neurological disease.

4.2 Study protocols

In studies I–II, three blood samples per patient were collected during the acute phase of the disease. The first sample was obtained as early as possible on admission, 2–9 (median 6) days after the onset of fever. The second sample was obtained 3–10 (median 7) days and the third 6–13 (median 10) days after the onset of fever. The fourth sample for the study was drawn at full recovery (ranging 32–54 days after the onset of fever, median 43 days). Citrate-anticoagulated (109 mM sodium citrate) samples were centrifuged at 1500 g for 20 min and the separated plasma samples were frozen at -70°C. Prior to analyses the defrozen samples were recentrifuged at 2500 g for 15 min.

In study III, the median number of plasma samples taken from the patients during the hospital stay was 4 (range 1–6). Again, the first sample was obtained on admission, 2–16 (median 5) days after the onset of fever, and the second 4–16 (median 6) days after the onset of fever. The third sample was taken 6–17 (median 8) days after the onset of fever and the fourth 6–14 (median 9) days after the onset of fever. The fifth sample was drawn 7–11 (median 10) and the sixth 13–18 (median 16) days after the onset of

fever. The last study sample was taken at full recovery, 18–55 (median 38) days after the onset of fever. The set of samples for SC5b-9 analyses were stored at -70°C and the set for C3/C4 analyses at -20°C.

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

Basic laboratory parameters determined at the Laboratory Centre of Pirkanmaa Hospital District together with clinical data were retrieved from patient charts for all four studies. Plasma IL-6 measurements were made on three consecutive mornings starting from the first morning of the hospital stay. For the patients in studies I–II treatment with plasma transfusions or platelets to correct possible coagulopathy as well as treatment with heparin were noted. In study III, all chest radiographs were studied retrospectively by an experienced radiologist.

4.3 Controls

In studies I–III, the samples taken in the recovery phase served as controls. In the case of study IV, data on the allele frequencies of the platelet antigens, VWF and plasminogen activator inhibitor (PAI-1) in the general population in the Pirkanmaa region are missing. This should not, however, be interpreted as a major shortcoming of study IV, since the focus of the study was on the severity of the disease in hospital-treated patients, not on the prevalence of the disease.

4.4 Methods

4.4.1 Serological diagnosis of PUUV infection

The specific serological diagnosis of acute PUUV infection was based on an IgM-capture EIA and PUUV Sotkamo strain full-length NP expressed by using the baculovirus system in Sf9 insect cells (Vapalahti et al. 1996). In comparison with various other protocols, the assay showed optimal sensitivity and specificity (Sjölander KB et al. 1997). The blood sample for the assay was taken in the beginning of the hospital stay, and the analyses were carried out at the Department of Virology in the University of Helsinki.

4.4.2 Basic laboratory tests and assessment of clinical disease

During the hospital stay, basic laboratory tests such as complete blood count (CBC), plasma C-reactive protein (CRP) and serum potassium and creatinine concentrations were carried out according to the clinical needs of the patients. Determinations were

made at the Laboratory Centre of Pirkanmaa Hospital District using standard methods.

Unfortunately there is not available any clinical score or grading system to assess the severity of hantavirus infection. Probably the most objective tool to measure the severity of hantavirus disease is the length of hospital stay. Widely accepted indicators of severe hantavirus disease include impaired renal function as measured by elevated serum creatinine concentration and need of transient hemodialysis treatment, hemorrhagic problems, clinical shock and death.

4.4.3 Biomarkers of coagulation and fibrinolysis (study I)

Laboratory analysis included PT expressed as % (PT%, Nycotest PT®, Axis-Shield PoC As), APTT (Actin FSL®, Siemens Healthcare Diagnostics) and TT (Siemens Healthcare Diagnostics). Fibrinogen was measured with a modification of the Clauss method (Multifibren® U, Siemens Healthcare Diagnostics) and D-dimer with an immunoturbidimetric assay (Tina-quant D-Dimer®, Roche Diagnostics). Prothrombin fragments (F1+2) were measured by an enzyme immunoassay (Enzygnost® F1+2, monoclonal, Siemens Healthcare Diagnostics). The reference values for PT% were 70–130%, APTT 23–33 s, TT 17–25 s, fibrinogen 1.7–4.0 g/l, D-dimer ≤ 0.5 mg/l and F1+2 69–229 pmol/l. The protein S free antigen (PS) level was determined by an automated latex ligand immunoassay (Instrumentation Laboratory), reference values being 66–158% for males and 50–177% for females. AT and protein C (PC) activities were both determined by chromogenic assays (Berichrom®Antithrombin III and Berichrom®Protein C, Siemens Healthcare Diagnostics), reference values being 84–108% and 74–141%, respectively. All laboratory analyses were performed in the Laboratory of Helsinki University Central Hospital.

4.4.3.1 Scoring for disseminated intravascular coagulopathy

The DIC scoring system by ISTH was used to evaluate individual patients (Taylor et al. 2001, Table 3). The most abnormal value for each variable was chosen for scoring based on the three acute assessments.

4.4.4 Platelet ligands and acute phase reactants (study II)

The activity of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13) was determined by immunochemical detection of its proteolytic target, i.e. VWF (Technozym Elisa, Technoclone). Factor VIII activity (FVIII:C) was determined by one-stage clotting assay (Pathromtin SL and Coagulation Factor

VIII Deficient Plasma, Siemens Healthcare Diagnostics). VWF antigen (VWF:Ag) and VWF:RCo were measured using the BCS XP analyser with BC VWF Reagent and VWFAg Latex Reagent (Siemens Healthcare Diagnostics). Fibrinogen was measured with a modification of the Clauss method (Multifibren® U, Siemens Healthcare Diagnostics). Analyses of ADAMTS13, FVIII:C, VWF:Ag, VWF:RCo and fibrinogen were made in the Laboratory of Helsinki University Central Hospital. Fibronectin concentration was determined at room temperature by enzyme-linked immunosorbent assay (human Fibronectin ELISA, Bender MedSystems) at the Haartman Institute. Plasma IL-6 concentrations were determined using a commercially available ELISA (PeliKine Compact ™ human IL-6 kit, Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service) with a detection limit of 0.4 pg/ml.

4.4.5 Complement system (study III)

Complement analyses were carried out at the Haartman Institute and in the Laboratory of Helsinki University Central Hospital. Plasma complement component C3 and C4 levels were measured by nephelometry (Dade Behring) and SC5b-9 using an ELISA kit (Quidel). Some patient samples did not meet the quality-control criteria for measurements of C3, C4 and SC5b-9 levels defined by the manufacturer. These samples were thus excluded from the respective analyses.

4.4.6 Genotyping (study IV)

The gene polymorphisms of GP IIIa (HPA-1) T>C (rs5918) and GP Ib (HPA-2) C>T (rs6065) were genotyped with Assay-By-Design from Applied Biosystems under standard conditions using the ABI Prism 7900HT Sequence Detection System (Taqman, Applied Biosystems). The GP Ia (HPA-5) T>C (rs1126643) and the VWF A>G (rs1063856) were genotyped as previously described (Kekomäki et al. 1995 and Kunkel et al. 1990, respectively). Genotyping of the GPVI T>C (rs1613662) was performed with minor modifications (Lepäntalo et al. 2006). For PAI-1 A>G (rs2227631) a commercial kit from Applied Biosystems was used. The distributions of all SNPs did not deviate from the Hardy–Weinberg equation.

4.4.7 Statistical methods

In studies I–III, the most abnormal value of each continuous variable measured during the acute phase of NE was designated the minimum or maxium value. In study I, the highest values of APTT, F1+2 and D-dimer and the lowest values of platelet count, PT, TT, fibrinogen, AT, PC and PS were used. In study II, the lowest of three ADAMTS13

and fibronectin values and platelet counts and the highest values of FVIII:C, VWF:Ag, VWF:RCo and fibrinogen were taken. In study III, the highest value of SC5b-9 was chosen to respresent the peak of complement activation, and the lowest values of C3 and C4 were chosen to represent the consumption during the acute phase of NE.

In studies I–III, the change between the maximum or the minimum and the control value (the fourth sample) was calculated. Means (± standard deviations) and medians (ranges) were provided. To evaluate changes for each variable between the acute and the control phase, paired samples *t*-test or Wilcoxon's test was used. Relationships between continuous variables were examined using Pearson's or the Spearman rank correlation coefficient. Comparisons between the groups were based on the Mann-Whitney U test for numerical and χ^2 test for categorical data.

In study IV, allele frequencies were counted and patients were divided into groups of carriers and non-carriers of the rarer (minor) allele. Comparisons between the groups were based on Mann-Whitney U or Kruskal-Wallis test for numerical and χ^2 or Fisher's exact test for categorical data.

In all four studies, the limit of significance was set at 0.05 (2-tailed). Computation was carried out using SPSS for Windows statistical software (version 7.5 in studies I–II, version 18 in study III and version 14 in study IV).

4.5 Ethical considerations

Written informed consent was obtained from all patients. The study design was approved by the Ethics Committee of Tampere University Hospital.

5 RESULTS

5.1 The clinical features (studies I–IV)

In all four studies, all patients were suffering from clinically typical acute PUUV infection. All 185 patients recovered.

In studies I–II, the most prominent symptoms on admission were: fever 100% (n=19), nausea and/or vomiting 63% (n=12), headache 47% (n=9), blurred vision 32% (n=6) and abdominal or back pain 16% (n=3). During the hospital stay only minor bleeding events occurred: two patients suffered from epistaxis and one from minor conjunctival bleed. One of the three patients who needed transient hemodialysis treatment showed oozing at the base of the central venous catheter for several days and received a platelet transfusion of two units. The patients with minor bleeding symptoms had IL-6 levels, leukocyte counts and CRP values higher than those without (p=0.007, p=0.019, for the last two, respectively). Five patients were treated with low-molecular-weight heparin for a few days either for thromboprophylaxis or maintenance of hemodialysis.

Four out of 61 (7%) patients participating in study III needed transient hemodialysis. In study IV, three out of 172 patients (2%) were in clinical shock at the time of admission. Seven patients (4%) needed transient hemodialysis treatment.

Basic clinical and laboratory findings in patients included in studies I–IV are presented in Table 4.

Table 4. *Basic clinical and laboratory findings in patients participating in studies I–IV.*

Variable	Studies I–II	Study III	Study IV
Length of hospital stay (days)	7 (3–15)	6 (2–15)	6 (2–15)
Diur $_{min}$ (ml)	880 (40–4770)	1620 (50–5800)	1450 (50–7000)
Change in weight (kg)*	3.2 (0.2–12.0)	2.7 (0–12.0)	2.1 (0–12)
Hematocrit $_{min}$	0.36 (0.28–0.45)	0.36 (0.25–0.43)	0.36 (0.25–0.46)
Leukocyte count $_{max}$ (x10^9/l)	11.8 (7.3–23.2)	9.9 (3.9–31.2)	10.0 (3.9–31.2)
Platelet count $_{min}$ (x10^9/l)	75 (13–238)	67 (0–238)	62 (3–238)
S-Creatinine $_{max}$ (µmol/l)	321 (74–1285)	175 (65–1285)	185 (51–1499)
P-CRP $_{max}$ (mg/ml)	61 (6–198)	69 (17–269)	75 (11–269)
P-Interleukin 6 $_{max}$ (pg/ml)	16.7 (3.6–96.6)[1]	11.7 (1.3–96.6)[2]	14.5 (1.3–107)[3]

Diur=daily urinary output, min=minimum, max=maximum, S=serum, P=plasma, CRP=C reactive protein. *Change in weight reflects the amount of fluid accumulated in the body during the oliguric phase of the disease. [1] n=19, [2] n=48, [3] n=118. The values represent median (range). Reference values: hematocrit 0.35–0.50 for males and 0.35–0.46 for females, leukocyte count 3.4–8.2 x 10^9/l, platelet count 150–360 x10^9 /l, S-creatinine <105 µmol/l for males and <95 µmol/l for females, P-CRP <10 mg/l.

5.2 Platelet ligands, coagulation and fibrinolysis (studies I–II)

Laboratory markers of endothelial cell activation, coagulation and fibrinolysis during the acute phase of PUUV infection and at recovery are presented in Table 5.

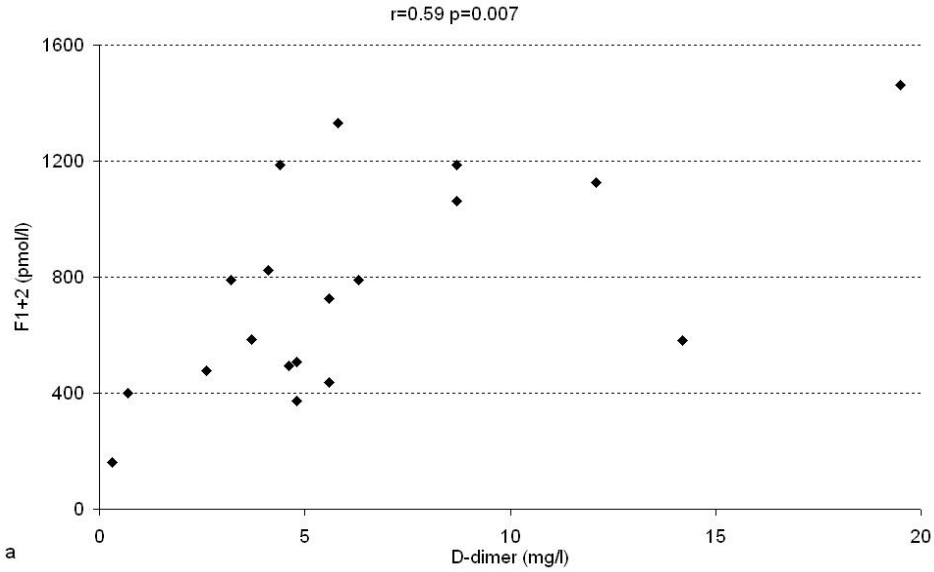
In study I, a markedly increased D-dimer during the acute phase of the disease was a prominent finding. The D-dimer level was 24-fold higher acutely compared with the control (Figure 1a in original publication I). F1+2 also increased 3.4-fold in the acute phase compared with recovery (Figure 1b in original publication I). D-dimer and F1+2 correlated positively (Figure 6a). Both remained within normal limits in only one patient.

Table 5. *Laboratory markers of coagulation, fibrinolysis and endothelial cell activation during the acute phase and at recovery in 19 patients with Puumala hantavirus infection (studies I–II).*

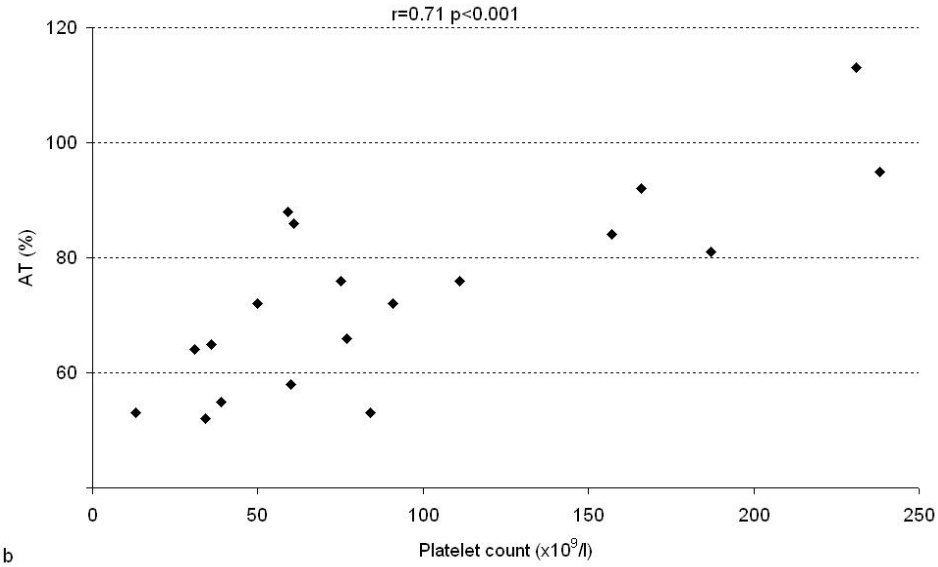
Variable	Reference values	Acute phase	Recovery phase	p
APTT max (s)	23–33	34 (29–47)	29 (26–34)	<0.001
Prothrombin time min (%)	70–130	84 ± 23	119 ± 31	<0.001
Thrombin time min (s)	17–25	16 ± 1.6	19 ± 1.9	<0.001
Fibrinogen min (g/l)	1.7–4.0	3.6 ± 1.2	3.2 ± 0.8	0.14
F1+2 max (pmol/l)	69–229	726 (160–1461)	213 (95–351)	<0.001
D-dimer max (mg/l)	≤0.5	4.8 (0.3–19.5)	0.2 (0.1–1.4)	<0.001
Antithrombin activity min (%)	84–108	72 (52–113)	95 (64–119)	<0.001
Protein C activity min (%)	74–141	61 (32–121)	106 (64–185)	<0.001
Protein S free antigen min (%)	66–158	60 (19–90)	92 (68–121)	<0.001
VWF:Ag max (%)	51–169	252 (113–459)	88 (39–192)	<0.001
VWF:RCo max (%)	44–183	267±112	98±39	<0.001
FVIII:C max (%)	52–148	118 (56–173)	88 (39–192)	0.002
Fibrinogen max (g/l)	1.7–4.0	5.0±1.3	3.2±0.9	<0.001
ADAMTS13 max (%)	40–130	56 (34–90)	63 (45–85)	0.003
Fibronectin (μg/ml)	117–338	221 (114–350)	330 (138–928)	0.001

Max=maximum, min=minimum, APTT=activated partial thromboplastin time, F1+2=prothrombin fragments, VWF:Ag=von Willebrand factor antigen, VWF:RCo=VWF ristocetin cofactor activity, FVIII:C=factor VIII activity, ADAMTS13=a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13 activity. The values represent median (range) or mean ± SD.

Figure 6. *Correlations between (a) D-dimer and prothrombin fragments (F1+2), and platelet count and (b) antithrombin (AT), (c) protein C (PC) and (d) protein S free antigen (PS) in 19 patients with Puumala hantavirus infection.*

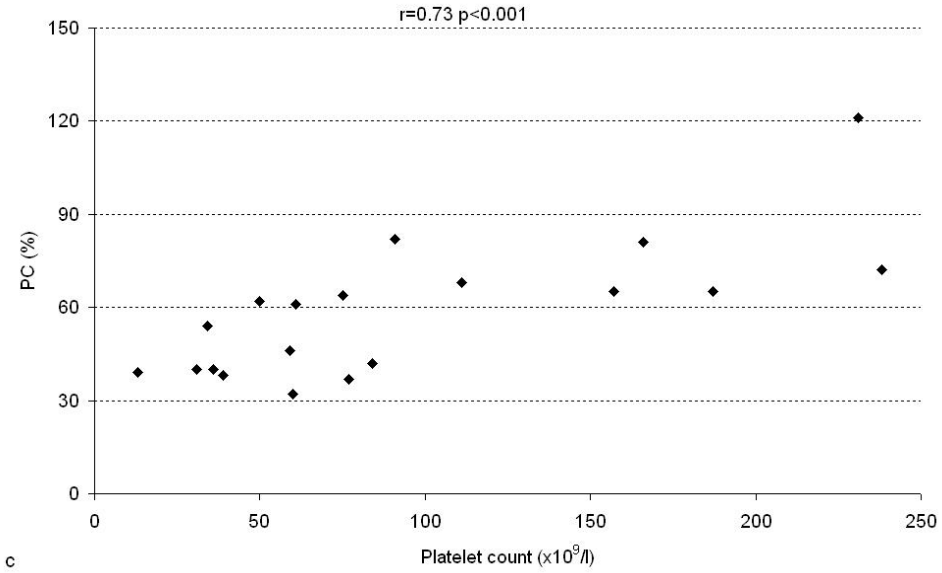


a

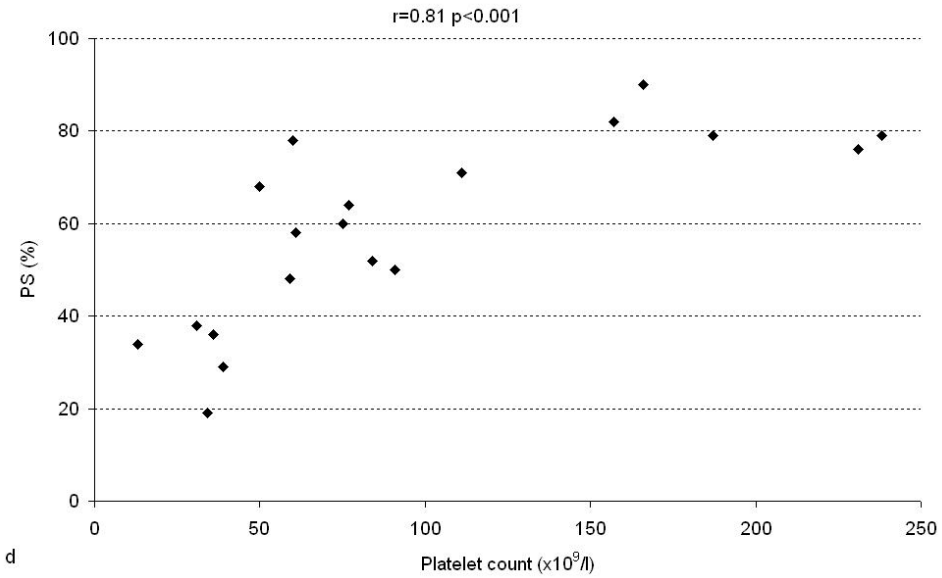


b

Figure 6...


r=0.73 p<0.001
PC (%)
Platelet count (x10^9/l)
0
30
60
90
120
150
0
50
100
150
200
250

c


r=0.81 p<0.001
PS (%)
Platelet count (x10^9/l)
0
20
40
60
80
100
0
50
100
150
200
250

d

Acutely, the levels of natural anticoagulants PC and AT (Figures 1c and 1d in original publication I) as well as PS were low and TT was shortened ($p<0.001$ for all) compared with the recovery phase. APTT was prolonged and PT% decreased ($p<0.001$ for both) compared with recovery. Among the most distinct of the number of correlations found in study I were the negative correlation between CRP and PC ($r=-0.58$, $p=0.009$) and the positive correlation between PC and AT ($r=0.73$, $p<0.001$).

In study II, the major platelet ligands VWF, fibrinogen and fibronectin were all affected in the acute phase of the infection (Figures 1a, 1b and 1d in original publication II). VWF:Ag and VWF:RCo levels were nearly threefold higher during the acute compared with the recovery phase, correlating well with each other ($r=0.94$, $p<0.001$). Only a slight increase in FVIII:C was noted acutely, and VWF levels did not correlate with FVIII:C. FVIII:C correlated with the maximum fibrinogen level ($r=0.589$, $p=0.008$), and this level was 1.6-fold higher during the acute compared with the recovery phase. ADAMTS13 activity as well as fibronectin concentration were lower in the acute phase compared with recovery ($p=0.003$ and $p=0.001$, respectively; Figures 1c and 1d in original publication II).

The reduced blood platelet count seen during the acute phase correlated with several variables. Especially strong correlations were observed between platelet count and AT and PC activities and PS (Figures 6b, 6c and 6d, respectively). Platelet counts correlated positively with minimum fibrinogen concentrations ($r=0.72$, $p<0.001$, study I) and negatively with maximum fibrinogen concentrations ($r=-0.468$, $p=0.043$, study II).

Renal function impairment, as assessed by maximum serum creatinine concentration or minimum daily urinary output, did not correlate with the platelet ligands, endothelial markers or coagulation variables, except for the shortening of thrombin time ($r=-0.59$, $p=0.007$ and $r= 0.53$, $p= 0.02$, respectively).

A number of correlations between the platelet ligands and ADAMTS13 (study II) and the variables measuring blood coagulation and fibrinolysis (study I) were noted. The plasma fibronectin level correlated negatively with D-dimer and F1+2 levels ($r=-0.49$, $p=0.03$ and $r=-0.71$, $p=0.001$, respectively) and positively with the PC level ($r=0.50$, $p=0.028$). Plasma fibrinogen maximum correlated inversely with thrombin time ($r=-0.64$, $p=0.003$). Positive correlations were found between plasma ADAMTS13 level and the natural anticoagulants AT and PC ($r=0.54$, $p=0.018$ and $r=0.52$ and $p=0.024$, respectively), negative correlations between ADAMTS13 and APTT and D-dimer ($r=-0.53$, $p=0.019$ and $r=-0.63$, $p=0.004$, respectively).

5.2.1 Disseminated intravascular coagulopathy

Five of the 19 patients (26%) reached at least 5 points in the ISTH score (Taylor et al. 2001, Table 3), indicating a diagnosis of DIC. In addition to the typical low platelet count and high D-dimer, all five had prolonged APTT and elevated F1+2 (Table 6). These five included the one who needed transient hemodialysis and experienced oozing at the site of the central venous catheter. However, there was no statistically significant difference between a positive DIC score and the length of hospitalization, maximum creatinine concentration, need for transient hemodialysis, or bleeding tendency (data not shown).

Table 6. *Data on five patients compatible with DIC according to the ISTH score (5 or over) (original article I, copyright Elsevier).*

Pt	Gender (F / M)	Age (years)	Dialysis	Platelet count min (x109/l)	PT min (%)	APTT max (s)	D-dimer max (mg/l)	Fibrinogen min (g/l)	F1+2 max (pmol/l)	DIC score
1	M	34	no	31	70	35	5.6	3.5	438	5
2	F	49	no	34	83	41	12.1	1.50	1125	5
3	M	30	no	39	40	38	19.5	1.8	726	6
4	M	36	no	36	76	46	6.0	2.4	1461	5
5	M	36	yes	13	60	40	3.2	2.5	789	5

DIC=disseminated intravascular coagulopathy, ISTH=the International Society of Thrombosis and Haemostasis, min=minimum, max=maximum, PT=prothrombin time, APTT=activated partial thromboplastin time, F1+2=prothrombin fragments. Reference values: platelet count 150–360 x 10^9/l, PT% 70–130%, APTT 23–33 s, D-dimer ≤ 0.5 mg/l, fibrinogen 1.7–4.0 g/l and F1+2 69–229 pmol/l.

Table 7 presents a comparison of coagulation results between DIC-negative and DIC-positive patients according to the ISTH score.

5.3 Markers of complement activation (study III)

The highest and lowest concentrations of SC5b-9 and C3, respectively, were found in the first or second sample in 50 out of 61 patients (82%) and in the third or fourth sample in the others. The lowest concentration of C4 was found in the first or second sample in 40 patients. Plasma SC5b-9 concentration in the acute phase was significantly higher than at recovery (Table 8). The acute-phase C3 level was decreased compared to that measured at recovery. The acute-phase C4 level was not significantly different from that measured at recovery.

Table 7. *Acute phase coagulation results of DIC-negative and DIC-positive patients according to the ISTH score (n=19).*

	Reference values	DIC negative	DIC positive	p
Platelet count min (x 109/l)	150–360	88 (50–238)	34 (13–39)	0.001
APTT max (s)	23–33	33 (29–47)	40 (35–46)	0.009
Prothrombin time min (%)	70–130	86 (64–140)	70 (40–83)	0.02
Fibrinogen min (g/l)	1.7–4.0	4.0 (2.7–6.7)	2.4 (1.5–3.5)	0.005
Antithrombin activity min (%)	84–108	78 (53–113)	55 (52–68)	0.01
Protein C activity min (%)	74–141	65 (32–121)	40 (38–54)	0.03
Protein S free antigen min (%)	66–158	70 (48–90)	36 (19–44)	0.001

DIC=disseminated intravascular coagulopathy, ISTH=the International Society of Thrombosis and Haemostasis, APTT=activated partial thromboplastin time, min=minimum, max=maximum. The values represent median (range).

Table 8. *The highest concentration of plasma SC5b-9 and lowest concentrations of plasma C3 and C4 in the acute phase and at recovery in 61 patients with PUUV infection.*

Variable	Acute-phase value	Recovery-phase value	p
SC5b-9 $_{max}$ (ng/ml)	493 (103–1034)	197 (100–522)	<0.001
C3 $_{min}$ (g/l)	1.26 (0.65–2.24)	1.47 (0.84–2.44)	<0.001
C4 $_{min}$ (g/l)	0.26 (0.08–0.52)	0.27 (0.09–1.1)	0.48

SC5b-9=the terminal complement complex SC5b-9, C3= complement component C3, C4=complement component C4, PUUV=Puumala hantavirus, max=maximum, min=minimum. The values represent median (range). Reference values: C3 0.71–1.41 g/l, C4 0.12–0.34 g/l.

Fourteen of those 50 patients (28%) who had chest X-ray taken had abnormal findings, including accumulation of pleural fluid (n=6) and atelectasis (n=12). The patients with chest X-ray abnormalities during the acute phase had significantly higher levels of SC5b-9 than those having normal images (median 637 vs 464 ng/ml, p=0.028), while these levels were no longer different at the time of recovery. The patients with pathological findings in chest X-ray had lower minimum C3 levels than those with normal X-ray, but the difference did not reach statistical significance. No differences were observed in C4 levels (data not shown).

5.3.1 Correlation with the clinical course of the disease

Acute-phase levels of SC5b-9 and C3 correlated significantly with a number of variables reflecting the clinical severity of PUUV infection. As to the highest SC5b-9, the strongest correlations were found with the length of hospital stay, the highest blood leukocyte count, and change in weight during hospital stay (r=0.46, r=0.43 and r=0.42, respectively, p<0.001 for all). The variables which showed the strongest correlation with the lowest C3 were the highest plasma IL-6, the lowest blood hematocrit, and the lowest serum sodium (r=-0.62, r=0.52 and r=0.44, respectively, p<0.001 for all). The leukocyte counts correlated inversely with C3 levels (r=-0.30, p=0.02), as well as with the length of hospital stay (r=-0.30, p=0.025). Acute-phase levels of C4 showed no significant correlation with any of the clinical or laboratory findings (data not shown).

5.3.2 Associations between the markers of complement activation (study III) and the findings in studies I–II

The level of C3 in the acute phase was associated with several of the laboratory variables in studies I–II measuring coagulation activity (n=19). An inverse correlation was noted between C3 and APTT (r=-0.70, p=0.003), and a positive correlation prevailed between C3 and all natural anticoagulants AT, PC and PS (r=0.76, p<0.001; r=0.53, p=0.025; r=0.64, p=0.004; respectively). No associations were found between SC5b-9 and C4 and the variables measuring the platelet ligands, blood coagulation and fibrinolysis.

5.4 Genetic polymorphisms of platelet antigens, VWF and PAI-1 (study IV)

Table 9 presents the genotype distributions and allele frequencies of GP IIIa (HPA-1), GP Ib (HPA-2), GP Ia (HPA-5), GP VI, VWF and PAI-1. Genotyping was successful in 162 out of 172 patients (94%) for HPA-1, in 169 patients (98%) for HPA-2, in all 172 patients for HPA-5, in 171 patients (99%) for GPVI, in 162 patients (94 %) for VWF and in 169 patients (98%) for PAI-1.

Table 9. *The genotype distributions and allele frequencies of platelet GP IIIa, GP Ib, GP Ia, GP VI, VWF and PAI-1 in 172 patients with acute PUUV infection (original article IV, copyright Elsevier).*

	Genotype %			Allele %	
Polymorphism	homozygote common	heterozygote	homozygote rare	common	rare
GP IIIa (HPA-1)	66 (TT)	32 (TC)	2 (CC)	82 (T)	18 (C)
GP Ib (HPA-2)	78 (CC)	21 (CT)	1 (TT)	88 (C)	12 (T)
GP Ia (HPA-5)	93 (TT)	7 (TC)	0 (CC)	96 (T)	4 (C)
GP VI T>C (rs1613662)	79 (TT)	19 (TC)	2 (CC)	89 (T)	11 (C)
VWF A>G (rs1063856)	68 (AA)	25 (AG)	7 (GG)	81 (A)	19 (G)
PAI-1 A>G (rs227631)	32 (AA)	53 (AG)	15 (GG)	59 (A)	41 (G)

GP=glycoprotein, VWF=von Willebrand factor, PAI-1=plasminogen activator inhibitor 1, PUUV=Puumala hantavirus, HPA=human platelet antigen.

5.4.1 Associations of polymorphisms with the clinical disease

Renal function impairment in the acute phase of the disease was associated with the rare G-allele of the PAI-1 gene. The carriers of the PAI-1 gene G-allele had a 1.7 times higher maximum level of creatinine than non-carriers (213 μmol/l vs. 122 μmol/l, p=0.01; Figure 1a in original publication IV). Also, the level of creatinine exceeded 300 μmol/l 1.7 times more often in G-carriers compared with non-carriers (38% vs. 22%, p=0.04). Of all PAI-1 G-carrier genotypes, GG-homozygotes had the highest maximum level of creatinine, followed by AG-heterozygotes and AA-homozygotes (249

μmol/l, 204 μmol/l and 122 μmol/l, respectively, p= 0.03). Six out of seven hemodialysis patients were G-allele carriers, and one of them was a GG-homozygote.

The rare G-allele of VWF A>G was associated with a lower level of maximum creatinine (131 μmol/l vs. 241 μmol/l, p=0.02). Accordingly, the level of creatinine exceeded 300 μmol/l less often in carriers of the VWF G-allele than in non-carriers (21% vs. 38%, p=0.03). The carriers of the rare GP VI gene C-allele had likewise maximum creatinine levels > 300 μmol/l less often than non-carriers (17% vs. 36%, p=0.03).

Analyses of genotype combinations revealed that patients (n=18, 11% of all patients) carrying the favorable VWF G-allele and not carrying the unfavorable PAI-1 G-allele had lower levels of creatinine compared with those not carrying the VWF G-allele and carrying the PAI-1 G-allele (n=73, 46% of all patients; 99 μmol/l vs. 250 μmol/l, respectively, p=0.002).

The platelet count nadir was lower in carriers of the rare GP Ia (HPA-5) gene C-allele compared to non-carriers (44 x 10^9/l vs. 64 x 10^9/l, p=0.02). The minor G-allele carriers of the PAI-1 gene proved to have a higher platelet count nadir compared with non-carriers (65 x 10^9/l vs. 54 x 10^9/l, p=0.04; Figure 1b in original publication IV).

The maximum level of plasma IL-6 was lower in carriers of the rare GP IIIa (HPA-1) C-allele than in non-carriers (13.4 pg/ml vs. 16.5 pg/ml, p=0.05). There were no significant associations with GP IIIa (HPA-1) genotype and CRP or leukocyte count (data not shown).

There were no statistically significant differences in the length of hospital stay, lowest blood pressure, maximum weight change, need for transient hemodialysis treatment or occurrence of shock compatible with polymorphisms studied here (data not shown).

The numbers of patients participating in studies I–II (n=19) and both studies III and IV (n=48) were quite small and the frequency of the minor GP Ia (HPA-5) C-allelle was low (4%). Thus the possible associations between the most interesting genetic polymorphisms, namely those of PAI-1,VWF and GP Ia and the findings concerning platelet ligands, blood coagulation, fibrinolysis (studies I–II) and complement activation (study III) could not be reliably assessed (data not shown).

6 DISCUSSION

6.1 Platelet ligands, thrombin formation and fibrinolysis (studies I–II)

The most prominent finding in study I was the 24-fold increase in D-dimer during the acute phase of the disease. This could indicate markedly upregulated fibrin degradation or turnover or reduced elimination of fibrin fragments, or both. Thrombin generation was not proportionate to fibrinolysis, as the increase in F1+2 level was 3-fold in the acute phase compared with recovery. Interestingly, increased thrombin formation as indicated by elevated F1+2 appeared to correlate with the length of hospital stay, which is an objective assessment of disease severity. Despite the laboratory abnormalities, a beneficial clinical balance between prothrombotic and fibrinolytic activities prevailed, as indicated by the lack of overt clinical coagulation problems.

Endothelial activation is thought to play a role in the pathogenesis of hantavirus infection (Cosgriff 1991, Cosgriff and Lewis 1991), and endothelial injury could contribute to the increase of thrombin formation and fibrinolysis in the acute phase of NE (Lippi et al. 2008). Platelet ligands VWF, fibrinogen and fibronectin carried by platelets, mediate platelet-platelet interactions and are implicated in the acute phase reactions and endothelial activation. Interestingly, all of these molecules are ligands of $\alpha IIb\beta_3$, which contains the β_3 integrin receptor for the pathogenic hantaviruses (Gavrilovskaya et al. 1999). In the context of endothelial activation and increased thrombin formation and fibrinolysis in acute NE, the determinations of platelet ligands WFW, fibrinogen and fibronectin (study II) were thus of interest.

The levels of VWF:Ag and VWF:RCo were increased almost three-fold acutely compared with the recovery phase. As VWF is produced by the endothelium and megakaryocytes and carried in the α-granules of circulating platelets, an increase in VWF level could imply endothelial cell injury and platelet activation with granule release. These mechanisms of enhanced platelet adhesion and activation could result in platelet consumption and thrombocytopenia, as has been described in dengue virus infection (Basuki 2003, Sosothikul et al. 2007). In study II, the increase in FVIII did not quite follow VWF levels and the ratios between VWF:Ag and FVIII in the acute phase and at recovery differed markedly. This could be explained by increased thrombin formation in the acute phase of the disease, as FVIII degrades rapidly after release from its carrier molecule VWF by the action of thrombin (Sadler 1998). Other possible mechanisms

include binding of FVIII to platelet-derived microparticles stimulated by thrombin or the complement proteins C5b-9 (Wiedemer et al. 1986, Gilbert et al. 1991). The observation of only a modest increase in FVIII during the acute phase of PUUV infection is compatible with findings in a study on dengue virus, in which reduced levels of coagulation factors were associated with low platelet count and the clinical severity of the disease (Srichaikul et al. 2000).

The maximum plasma concentration of fibrinogen was 1.6-fold higher acutely compared with the recovery phase. This could be a consequence of platelet activation and granule release, an assumption supported by the negative correlation between plasma fibrinogen and platelet count. Elevated fibrinogen could also reflect an acute phase reaction strong enough to outweigh the consumption due to the ongoing coagulation activity, as observed during the acute phase of PUUV-HFRS in study I and HTNV-HFRS in earlier East-Asian studies (Lee 1987, Xiang et al. 1990).

The plasma fibronectin level decreased significantly during the acute phase of the disease compared with recovery, an observation consistent with a previous report of diminished plasma fibronectin levels in sepsis (Ruiz Martin et al. 2004). These findings differ from a previously published study reporting increased plasma fibronectin levels in HTNV-HFRS (Han et al. 2010). As fibronectin binds to fibrinogen and fibrin at low temperatures and the temperature in which the tests were carried out was not provided, the discrepancy of the findings in these studies could well be explained by methodological aspects. Thrombin enhances the binding of fibronectin to platelets (Ginsberg et al. 1983), and diminished plasma fibronectin levels could reflect the binding of fibronectin to thrombin-activated platelets and consumption during coagulation activity shown to be ongoing in study I. This concept is supported by the inverse correlation between plasma fibronectin level and the indicators of thrombin formation (F1+2) and fibrinolysis (D-dimer) as well as the positive correlation between fibronectin and the natural anticoagulant PC.

While the increase in plasma fibrinolytic activity as reflected by the 24-fold D-dimer in the acute compared with the recovery phase was substantial, the bleeding problems were few and minor. A number of procoagulant changes which probably helped to retain a beneficial clinical balance could be noted. The diminished activities of the natural anticoagulants AT, PC and PS were marked, but also rapidly reversed after the acute phase of the disease. VWF enhances early platelet adhesion and aggregation during both physiological and pathological thrombosis (Sadler 1998), and an increase in plasma VWF level shifts the balance in the procoagulant direction. Fibrinogen contributes to thrombus stability by anchoring the thrombi to the vessel wall and individual platelets to the thrombus (Denis et al. 2007), and an increase in plasma fibrinogen level ensures the sufficient availability of fibrinogen needed to maintain hemostasis during the ongoing fibrinolytic activity. All these procoagulant changes are likely to contribute to the maintenance of hemostasis in spite of the decreasing platelet count and markedly increased fibrinolytic activity.

ADAMTS13 is an enzyme specifically involved in the cleavage of high-molecular-weight VWF. It is known to play a role in the pathogenesis of thrombotic thrombocytopenic purpura (TTP), a thrombotic microangiopathy characterized by thrombocytopenia, hemolytic anemia and ischemic organ failure caused by thrombotic occlusions in the arterioles. Since thrombocytopenia and capillary injury are clinical hallmarks of acute NE and variable neurological symptoms are also encountered, the determination of ADAMTS13 activity in the acute phase of the disease was of interest. The finding of modestly reduced ADAMTS13 activity during the acute phase of NE is in accord with the reports implying that ADAMTS13 activity is diminished in dengue virus infection (Sosothikul et al. 2007) as well as in several other conditions (Kavakli et al. 2002). The positive correlations between ADAMTS13 and the natural anticoagulants AT and PC as well as the negative correlation between ADAMTS13 and APTT and D-dimer further point a role for endothelial injury in the pathogenesis of the enhanced coagulation and fibrinolysis encountered in the acute phase of NE.

The patients participating in studies I–II were clinically quite ill. Coagulation problems were minor, but three out of 19 (16%) needed transient hemodialysis. This figure is clearly higher than the 5% presented in the literature (Vapalahti et al. 2003). No association was found between the depth of thrombocytopenia and the degree of renal impairment. Neither the indicators of thrombin formation and fibrinlysis nor the increased consumption of coagulation inhibitors provided tools to predict the severity of kidney injury in this study. The only association to be found was that between creatinine concentration and thrombin time. Thrombocytopenia and short thrombin time could imply microvesiculation, i.e. membrane shedding with enhanced coagulation activity. This mechanism has been described in several conditions, including severe inflammation, complement activation and immune-mediated thrombocytopenia induced by heparin or hepatitis (Warkentin et al. 1999, Peerschke et al. 2008, Burnier et al. 2009). The strong positive correlations between the platelet count and the natural anticoagulants AT, PC and PS suggest temporal interactions between the platelets, their activation and impaired coagulation inhibitors, compatible with endothelial injury or activation. The interaction of platelets with the endothelium, their activation and P-selectin expression could thus provide a mechanism of thrombocytopenia during PUUV infection, as has been described in other forms of viral inflammation (Wagner et al. 2003, Dole et al. 2005, Zahn et al. 2006, Iannacone et al. 2007). On the other hand, thrombocytopenia in hantavirus infection has also been considered more of a result of platelet inactivation (Cosgriff et al. 1991) and binding of hantaviruses and hantavirus-infected endothelial cells to quiescent platelets via β_3 integrin receptor (Gavrilovskaya et al. 2010).

The number of patients participating in studies I–II was small, but the findings were consistent. As is frequently the case in clinical studies, the timing of the samples overlapped slightly, since the patients sought medical assistance at different time intervals from the beginning of the disease. However, the findings of altered platelet ligands

and endothelial markers together with ongoing coagulation and fibrinolysis in the acute phase of NE were clear.

6.1.1 Findings suggestive of disseminated intravascular coagulopathy

To date there is still no specific treatment available for HFRS. Since even the most severely ill patients are treated symptomatically, a useful predictive tool for selecting those in need of early and intensive treatment would be particularly valuable. As earlier reports have suggested the presence of DIC in some patients with PUUV-HFRS, the ISTH score for overt DIC (Taylor et al. 2010, Table 3) was applied to grade the laboratory findings and determine the possible associations between the score and the clinical course of the disease.

In this study five of the 19 patients (26%) reached the score of at least five points indicative of DIC. Such an incidence is comparable to that (28%) observed in a recent Swedish study of 106 patients (Sundberg et al. 2011). Interestingly, only one of the 19 patients had minor problems with hemostasis and there were only two patients with abnormal PT. No association could be found between the positive score and the two most prominent findings in study I, namely elevated D-dimer and F1+2. The score was not associated with clinical variables, and it failed to predict the clinical course of the disease in this small group of PUUV patients. However, as the Swedish study showed, the ISTH score for overt DIC, including the fibrinogen/CRP–ratio, might provide a clinical tool with high negative predictive value (Sundberg et al. 2011).

6.2 Complement activation and association with disease severity (study III)

Study III showed that the complement system is activated through the alternative pathway during the acute phase of PUUV infection. The terminal complement complex SC5b-9 was elevated acutely compared with recovery, and the level was significantly higher in patients with chest X-ray abnormalities. As a sign of increased consumption, the level of complement component C3 was decreased acutely. The level was lower, though not significantly, in patients with X-ray abnormalities compared to those with normal X-ray.

Capillary leakage has been suggested to contribute to the lung disease in HCPS and HFRS (Kanerva et al. 1998a), and immunological mechanisms may be partially responsible for the disturbed endothelial function. SC5b-9 is capable of increasing endothelial permeability by ligating β_3 integrin, the receptor for the pathogenic hantaviruses, which may result in elevated hydraulic conductivity (Tsukada et al. 1995). SC5b-9 releases bradykinin and platelet-activating factor, thus promoting endothelial permeability (Bossi et al. 2004). Also, the non-soluble equivalent of SC5b-9, the

MAC complex, is known to trigger cellular reactions and production of inflammatory cytokines capable of altering endothelial function (Morgan BP 1999). Obviously, the findings in study III do not prove a causal relationship between any of these mechanisms and lung disease in NE, since no experimental evidence is provided.

The markers of complement system activation, elevated SC5b-9 and decreased C3, proved to be associated with several variables reflecting the clinical disease. The length of hospital treatment, which is an objective means of measuring disease severity, correlated with both markers, implying complement activation through the alternative pathway. Interestingly, an inverse correlation was found between the plasma levels of C3 and IL-6, the latter known to be a useful tool in the evaluation of disease severity in acute PUUV infection (Outinen et al. 2010). A previous study (Paakkala et al. 2000) on complement activation and NE linked classical pathway activation to more severe disease. However, as that study included only six patients with mainly classical route activation, the common occurrence of complement activation (23 out of 25 patients) could equally be interpreted as the major finding in that study. These findings of complement activation associated with variables reflecting the clinical disease are in line with those indicating that complement activation contributes to a more severe clinical outcome in dengue fever and H1N1 pandemic influenza (Nascimento et al. 2009, Monsalvo et al. 2011).

The molecular intercommunication between complement system and blood coagulation and fibrinolysis is extensive, and the 19 patients participating in all studies I–III provided an opportunity to study possible associations between the biomarkers active in these cascades. SC5b-9 is able to catalyze prothrombin cleavage to thrombin (Wiedemer et al. 1986). Thrombin is capable of generating the complement activation products C3a and C5a (Huber-Lang et al. 2006, Amara et al. 2010), and C5a, in turn, possesses fibrinolytic activity (Wojta et al. 2002). Increased levels of SC5b-9, F1+2 and D-dimer were the major findigs in studies I–III, indicating the activation of both complement and blood coagulation and fibrinolysis. In this series no association between these biomarkers could be established, a finding that might be explained by the small number of patients. However, the associations between C3 and APTT and natural anticoagulants AT, PC and PS all imply ongoing simultaneous activity in the alternative pathway of the complement system and blood coagulation in PUUV-HFRS. This is in line with the previous reports establishing associations between decreased C3 levels and coagulation factors in HTNV-HFRS (Lee 1987) and stating that activation through the alternative route is a frequent finding in PUUV-HFRS (Paakkala et al. 2000).

To conclude, the findings in study III indicate that complement is activated through the alternative pathway in acute PUUV infection and the activation is associated with disease severity especially in the lungs. The simultaneous loss of natural anticoagulants emphasizes the molecular interaction between the complement system and blood coagulation and fibrinolysis.

6.3 Polymorphisms of PAI-1 and platelet GP Ia and clinical course of the disease (study IV)

The platelet count as well as the platelet function and blood coagulation and fibrinolysis are clearly altered in hantavirus infection. However, studies on the genetic polymorphisms affecting platelet functions and factors involved in blood coagulation and fibrinolysis in hantavirus infection are limited to one (Liu et al. 2009a). In study IV here, the aim was to establish possible associations between the polymorphisms of the main antigens involved in platelet activation and aggregation (GPs IIIa, Ib, Ia and VI) and VWF and PAI-1, and the clinical course of Puumala hantavirus disease.

The most prominent finding in study IV was the association of the PAI-1 gene minor G-allele with the major outcome of the disease, namely kidney function. The maximum level of creatinine and the proportion of patients having creatinine values > 300 μmol/l were higher among those carrying the G-allele compared with non-carriers. The finding was further emphasized by observations of GG-homozygotes having the highest creatinine levels and the G-allele carriers being over-represented among the patients who needed transient hemodialysis. In addition, the minor G-allele of the VWF gene (rs1063856) and the minor C-allele of GP VI (rs1613662) offered some protection of renal function in terms of serum creatinine level. The other clinical hallmark of PUUV infection, low platelet count, was associated with the low frequency C-allele of GP Ia (HPA-5).

PAI-1 is secreted by a variety of cells, including platelets and endothelial cells, both intimately involved in the pathogenesis of hantavirus infection, and PAI-1 is the primary physiological regulator of plasminogen activators and fibrinolysis. PAI-1 polypeptide is encoded by the SERPINE1 (SERine Proteinase Inhibitor, clade E, member 1) gene on chromosome 7q21.3-q2 (Koch et al. 2010). Association of the 4G/5G polymorphism (rs1799889) with increased PAI-1 levels of 4G/5G carriers has been suggested in several studies, and the SNP rs2227631 investigated in study IV is very close to the 4G/5G polymorphism (rs1799889), and has a relatively high correlation with 4G/5G (Kathiresan et al. 2005). The 4G-allele seems to be associated with atherosclerotic diseases of the coronary arteries (Koch et al. 2010) and reduced kidney graft survival (Rerolle et al. 2008). Moreover, elevated levels of PAI-1 have been related to poor outcome in conditions such as dengue virus infection, pneumonia, sepsis and meningococcal disease (Wills et al. 2002, Hermans et al. 2005, Mairuhu et al. 2005, Madach et al. 2010). Unfortunately the plasma levels of PAI-1 were not measured in study IV, and there are no published data on the plasma level and role of PAI-1 in hantavirus infection.

High plasma VWF levels during the acute phase of PUUV infection were reported in study II, all the same patients being included in study IV. Moreover, the A>G polymorphism of the VWF gene (rs1063856) in exon 18 of chromosome 12 investigated in study IV, is known to be associated with high VWF levels. It is also close to SNP

rs10638857, which is associated with VWF levels in healthy subjects (Lacquemant et al. 2000, Klemm et al. 2005, Campos et al. 2011). However, even if the VWF gene is highly polymorphic, genetic factors are responsible for only up to 66% of the variation in plasma VWF levels (Campos et al. 2011). Since the VWF levels in the minor G-allele (rs1063856) carriers and non-carriers were not compared in study IV, it is not possible to conclude whether or not the favorable effect of this polymorphism on kidney function depends on modulation of plasma VWF levels.

Polymorphisms of both collagen receptors GP VI and GP Ia (HPA-5) were associated with clinical disease, the minor C-allele of GP VI offering some protection of renal function and the minor C-allele of GP Ia correlating with low platelet count nadir. Low-frequency allele homozygotes of GP VI such as the CC genotype of T>C rs1613662 are associated with reduced platelet activation and responses to collagen when compared with high-frequency allele homozygotes (Joutsi-Korhonen et al. 2003). As the platelet functions were not studied here, it can not be concluded whether these effects on clinical disease depended on modulation of the polymorphisms on platelet activation, procoagulant activity and the formation of hemostatic plug contributed by collagen receptors.

The GP IIbIIIa complex binds platelet ligands VWF, fibrinogen and fibronectin, all shown to be altered in study II. The receptor for the pathogenic hantaviruses, subunit β_3 integrin (IIIa), is carried by GP IIbIIIa. Carriers of the low-frequency C-allele of GP IIIa (HPA-1) had slightly lower levels of plasma IL-6, a variable correlating with disease severity in acute PUUV infection. This finding is in line with an earlier report stating that HPA-1 polymorphism of GP IIIa is not associated with severe disease in HTNV infection (Liu et al. 2009a).

Here, for the first time, genetic polymorphisms related to platelet activation, blood coagulation and fibrinolysis are reported to have an impact on both kidney function and platelet counts in HFRS. The impact of the polymorphism of PAI-1, a major regulator of fibrinolysis, on kidney function is the major finding of the study.

6.4 Future considerations

The number of patients participating in studies I–II was somewhat limited, but the findings of activated endothelium, ongoing coagulation activity and fibrinolysis were clear. Complement was shown to be activated via the alternative route in study III, and moreover, to be associated with the clinical disease. As evidence of molecular interaction between the complement system and blood coagulation and fibrinolysis is accumulating, it would be of interest to expand studies I–II to include all 61 patients who participated in study III. Most important would be to investigate the markers of thrombin formation and fibrinolysis (prothrombin fragments F1+2 and D-dimer) in all patients in study III. Recent data suggest that coagulation/fibrinolysis proteases may

act as natural C3 and C5 convertases, and of particular therapeutic interest is the ability of the FXa inhibitors fondaparinux and enoxaparin to suppress the FXa-induced cleavage of C3 (Amara et al. 2010).

As previously discussed, several polymorphisms related to platelet aggregation and activation, blood coagulation and fibrinolysis were shown to be associated with the clinical disease during the acute phase of PUUV infection. To further clarify the role of these polymorphisms in the pathogenesis of PUUV infection, platelet functions and plasma levels of factors involved in blood coagulation and fibrinolysis should be measured in patients carrying and not carrying particular polymorphisms. The role of GPs and platelet immunology in PUUV-induced thrombocytopenia could be evaluated by detecting possible GP antibodies.

Microparticles (MP) derive from different cell types, including platelets, the main source of MPs, but also from monocytes, granulocytes, lymphocytes, erythrocytes and endothelial cells. MPs are released from the cells upon activation or apoptosis and when circulating in the blood provide an additional procoagulant phospholipid surface enabling the assembly of the clotting enzyme complexes and thrombin formation (Burnier et al. 2009). Elevated levels are encountered in several clinical conditions of bleeding and thrombotic disorders (for example, heparin-induced thrombocytopenia, antiphospholipid syndrome and TTP), cardiovascular diseases and infectious diseases (for example, sepsis, HIV infection and prion disease) (Nomura et al. 2008). However, challenges remain regarding the methods used in the determination of MPs, and their significance in various conditions remains to be further elucidated. The findings of thrombocytopenia and short thrombin time in study I could imply MP formation, and the association of thrombin time with kidney function provides a further stimulus to investigate MP formation. Moreover, the complement activation observed in study III suggests a possible role of MP formation in the pathogenesis of PUUV infection, as both SC5b-9 and thrombin stimulate MP formation (Peerschke et al. 2008, Burnier et al. 2009). MPs might also be a part of an explanation for the only modestly increased FVIII observed in study II, since FVIII may be bound to MPs (Gilbert et al. 1991).

Neutrophil extracellular traps (NET) are part of the innate immune response to infections. They form a meshwork of deoxyribonucleic acid fibers comprising histones and antimicrobial proteins which immobilize and kill microbes. NETs are formed inside the vasculature in both infectious and non-infectious diseases, and platelets enhance the ability of neutrophils to make NETs. NETs, in turn, promote platelet adhesion, aggregation and activation. They are important in the control of infection, yet in excess or at inappropriate locations they contribute to vascular toxicity, organ failure and death (Fuchs et al. 2010, Esmon et al. 2011). The literature on NETs in viral infections is so far very limited, but the decreased platelet count with ongoing coagulation activity and microvascular injury are suggestive of NET formation as a possible mechanism in hantavirus infection. Unfortunately, detection of NET formation still remains challenging.

7 SUMMARY AND CONCLUSIONS

The main findings in the present series can be summarized as follows:

I Enhanced thrombin formation and marked fibrinolysis characterize the acute phase of PUUV infection. The commonly seen thrombocytopenia is associated with decreased natural anticoagulants AT, PC and PS.

II Five out of 19 patients fulfilled the ISTH criteria for DIC. The scoring could not predict the outcome of patients in terms of the most prominent laboratory findings (elevated F1+2 and D-dimer) or the clinical variables.

III Platelet ligands VWF, fibrinogen and fibronectin are all altered during the acute phase of PUUV infection. FVIII does not quite follow the increase observed in VWF level, and the activity of ADAMTS13 is only modestly reduced.

IV The complement system becomes activated via the alternative pathway during the acute phase of PUUV infection. The level of activation is associated with the severity of the disease.

V Polymorphism of PAI-1 and platelet GP Ia are associated with renal function and thrombocytopenia in PUUV infection.

The findings of enhanced thrombin formation and especially fibrinolysis were conspicuous, and natural anticoagulants were markedly diminished. Nonetheless none of these variables was associated with disease severity, and also the ISTH score for DIC failed to predict clinical outcome in this small series of patients. The adhesive platelet ligands and biomarkers related to endothelial activation and acute-phase reactants were altered, but none of them provided a prognostic clinical tool in the assessment of disease severity. On the other hand, complement activation via the alternative pathway was associated with several clinical and laboratory variables depicting disease severity. Especially pulmonary X-ray findings were more frequent in patients with high SC5b-9 levels and low C3 levels, which could be taken to signify a role of complement activation in capillary leakage causing pulmonary symptoms in PUUV infection.

For the first time, associations between several polymorphisms related to platelet activation, blood coagulation and fibrinolysis and severity of disease in acute hantavi-

rus infection were here investigated. The most prominent finding was the association between PAI-1, the major regulator of plasma fibrinolytic activity, and kidney function. This observation emphasizes the impact of the hemostatic system on the outcome of PUUV-HFRS, a form of hantavirus infection usually presenting with only modest hemostatic impairment.

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Outi Laine

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Regular Article

Enhanced thrombin formation and fibrinolysis during acute Puumala hantavirus infection

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ABSTRACT

Introduction: Nephropathia epidemica (NE) is a viral hemorrhagic fever with renal syndrome associated with thrombocytopenia and mild bleeding. We assessed activation of coagulation and fibrinolysis during the acute phase of NE.

Materials and methods: 19 hospital-treated patients were involved. Plasma levels of D-dimer, prothrombin fragments 1 + 2 (F1 + 2), activated partial thromboplastin time (APTT), prothrombin time (PT%), thrombin time (TT), fibrinogen, antithrombin (AT), protein S free antigen (PS), protein C (PC) and complete blood count (CBC) were measured three times during the acute phase and once at 32-54 days after the onset of fever (recovery phase). Laboratory abnormalities were evaluated by the disseminated intravascular coagulation (DIC) scoring advocated by the International Society of Thrombosis and Haemostasis (ISTH).

Results: APTT was prolonged and D-dimer and F1 + 2 increased during the acute phase of NE. AT, PC and PS decreased, and TT was shortened, all implying increased thrombin generation. Acutely F1 + 2 was 3.4-fold and D-dimer even 24-fold higher compared with the recovery phase (median 726 vs 213 pmol/l, and median 4.8 vs 0.2 mg/l, respectively, $p<0.001$ for both). Platelet count correlated with AT, PC, and PS ($r=0.73$, $r=0.81$, and $r=0.71$, respectively, $p<0.001$ for all) as well as with fibrinogen ($r=0.72$, $p<0.001$). Only five patients fulfilled the ISTH diagnosis of DIC.

Conclusions: During acute NE thrombocytopenia was associated with decreased natural anticoagulants, shortened thrombin time and enhanced fibrinolysis. Augmented thrombin formation and fibrinolysis characterize this hantavirus infection.

Introduction

Puumala virus (PUUV), a member of the *Hantavirus* genus in the *Bunyaviridae* family, is an enveloped RNA virus transmitted to humans by inhalation of aerosolized excreta of infected rodents. The natural host of PUUV is the bank vole, *Myodes glareolus,* which can be found throughout Europe with the exception of Mediterranean coastal regions and most of the Iberian Peninsula and Greece [1].

In humans hantaviruses cause a spectrum of illnesses which are often divided into two clinical diseases, hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. Acute PUUV infection, i.e. nephropathia epidemica (NE), is a mild form of HFRS. The disease may, however, in rare cases be fatal. In Finland most infections probably remain subclinical or undiagnosed, since only about 1000-2000 serological diagnoses are made annually despite a seroprevalence of 6% [2,3]. The clinical onset of the disease is characterized by high fever, headache, nausea and vomiting with abdominal and/or lower back pains [2]. Transient proteinuria, hematuria and rising serum creatinine levels indicate renal involvement and about 5% of hospital-treated patients require transient hemodialysis [4]. Pulmonary manifestation is also common in NE [5]. Although clear thrombocytopenia is encountered in the majority of NE patients, bleeding manifestations are usually mild. Petechiae, epistaxis, macroscopic hematuria and conjunctival bleeding have been reported [6]. Nuutinen and coworkers demonstrated mild gastrointestinal bleeding by gastroscopy in nearly all NE patients [7]. In

Abbreviations: NE, nephropathia epidemica; F1 + 2, prothrombin fragments; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; AT, antithrombin; PS, protein S free antigen; PC, protein C; CBC, complete blood count; DIC, disseminated intravascular coagulopathy; ISTH, International Society of Thrombosis and Haemostasis; PUUV, Puumala virus; HFRS, hemorrhagic fever with renal syndrome; HCPS, hantavirus cardiopulmonary syndrome; CRP, C-reactive protein, and HPA, human platelet alloantigen.

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fatal cases of NE hemorrhage of the pituitary gland and other organs has been reported [8–10].

Thrombocytopenia and bleeding tendency in NE are commonly considered signs of disseminated intravascular coagulopathy (DIC) [11,12]. However, so far data published on the activity and regulation of coagulation and fibrinolysis in NE remain sparse.

We aimed to evaluate different laboratory markers of coagulation and fibrinolysis during the acute phase of NE in association with the clinical severity of the disease. Further, we sought to grade the possible DIC during the acute phase of NE with the ISTH score.

Materials and methods

The study was carried out in Tampere University Hospital, University of Tampere Medical School, and Helsinki University Central Hospital, Finland. The patients were selected from those who participated in a previous larger prospective study basing on the availability of the laboratory samples. All patients came from the Pirkanmaa region and were hospitalized in Tampere University Hospital with serologically confirmed acute PUUV infection during the period from September 2000 to December 2002. The study group consisted of 19 patients (17 males), median age 38 years (range 30-64 years). Regarding medical history, two of the patients had a neurological disease (1 multiple sclerosis, 1 epilepsy) and one a chronic inflammatory bowel disease treated with mesalazine medication. There were patients with dyslipidemia (n = 2), coronary heart disease (n = 1), arterial hypertension (n = 1) and paroxysmal atrial fibrillation (n = 1). No patient was under immunosuppression or anticoagulation. Two patients used anti-platelet therapy (aspirin).

The patient charts were retrospectively reviewed to establish the length of hospital stay (days), daily urinary output (ml), need of transient hemodialysis treatment (no/yes) and signs of bleeding (no/yes). The treatment with platelet or plasma transfusions to correct the possible coagulopathy as well as the treatment with heparin were noted.

To assess the activation of coagulation and fibrinolysis, three blood samples per patient were collected during the acute phase of the disease. The first sample was obtained on admission as early as possible, 2-9 (median 6) days after the onset of fever. The second sample was obtained 3-10 (median 7) days, and the third 6-13 (median 10) days after the onset of fever. The last sample of the study, the fourth sample, was drawn at full recovery (ranging 32-54 days after the onset of fever, median 43 days), this representing a control sample. Citrate-anticoagulated (109 mM sodium citrate) samples were centrifuged at 1500 g for 20 min and separated plasma samples were frozen at -70 °C. Once defrosted the samples were recentrifuged at 2500 g for 15 min prior to analysis.

Laboratory analysis included plasma prothrombin time expressed as % (PT%, Nycotest PT®, Axis-Shield PoC As, Oslo, Norway), activated partial thromboplastin time (APTT, Actin FSL®, Siemens Healthcare Diagnostics, Marburg, Germany) and thrombin time (TT, Siemens Healthcare Diagnostics). Fibrinogen was measured with a modification of the Clauss method (Multifibren® U, Siemens Healthcare Diagnostics) and D-dimer with an immunoturbidimetric assay (Tina-quant D-Dimer®, Roche Diagnostics, Mannheim, Germany). Prothrombin fragments (F1 + 2) were measured by an enzyme immunoassay (Enzygnost® F1 + 2, monoclonal, Siemens Healthcare Diagnostics). The reference values for PT% were 70-130%, APTT 23-33 s, TT 17-25 s, fibrinogen 1.7-4.0 g/l, D-dimer ≤ 0.5 mg/l and F1 + 2 69-229 pmol/l. Protein S free antigen (PS) level was determined by an automated latex ligand immunoassay (Instrumentation Laboratory, Lexington, MA), reference values being 66-158% for males and 50-177% for females. Antithrombin (AT) and protein C (PC) activities were both determined by chromogenic assays (Berichrom®Antithrombin III and Berichrom®Protein C, Siemens Healthcare Diagnostics), reference values being 84-108% and 74-141%, respectively.

During the hospital stay, complete blood count (CBC), plasma C-reactive protein (CRP) and serum creatinine were taken according to the clinical needs of the patients, and determined at the Laboratory Centre of the Pirkanmaa Hospital District using standard methods. The reference values for serum creatinine were <105 μmol/l for males and <95 μmol/l for females and for CRP<10 mg/l. For platelet and leukocyte counts the reference values were 150-360 and 3.4-8.2 × 10^9/l, respectively. The reference values for blood hemoglobin were 134-167 g/l for males and 117-155 g/l for females.

The DIC scoring system by the International Society of Thrombosis and Haemostasis (ISTH) was used to evaluate individual patients [13]. The most abnormal value for each variable was chosen for scoring based on the three acute assessments.

Statistics

To analyse the data, the most abnormal value of each continuous variable measured during the acute phase of NE was designated the maximum or the minimum value. The change between the maximum or the minimum and the control value (the fourth sample) was calculated. Means (± standard deviations) and medians (ranges) were provided. To evaluate changes for each variable between the acute and the control phase, paired samples *t*-test or Wilcoxon's test was used. Relationships between continuous variables were examined using Pearson's or the Spearman rank correlation coefficient. Comparisons between the groups were based on the Mann-Whitney U test for the numerical and χ^2 test for the categorical data. The limit of significance was set at 0.05 (2-tailed). SPSS 7.5 was used for computation.

Results

Clinical and laboratory findings

All 19 patients presented with clinical characteristics typical of NE. The most prominent symptoms on admission were: fever 100% (n = 19), nausea and/or vomiting 63% (n = 12), headache 47% (n = 9), blurred vision 32% (n = 6) and abdominal or back pain 16% (n = 3). The median duration of hospitalization was 7 days (range 4-15 days). During the hospital stay only minor bleeding events occurred: two patients suffered from epistaxis and one from minor conjunctival bleed. The median lowest daily urinary output was 880 ml (range 40-3740 ml). Three of the 19 patients needed transient hemodialysis treatment; one of them showed oozing at the base of the central venous catheter for several days and received a platelet transfusion of two units. Five patients were treated with low molecular weight heparin for a few days either for thromboprophylaxis or maintenance of hemodialysis. All patients recovered.

During the acute phase of NE the lowest platelet count ranged from 13 to 238 × 10^9/l (median 75 × 10^9/l), and thrombocytopenia was found in 15 (79%) patients. However, 17 patients (89%) had their platelet count within normal values by the time of discharge. Seventeen patients had a leukocyte count higher than 10.0 × 10^9/l (median maximum 11.8 × 10^9/l, range 7.3 - 23.2 × 10^9/l), and 15 patients were anemic (median minimum hemoglobin 126 g/l, range 98 - 157 g/l). CRP was elevated in 17 patients (median maximum 61 mg/l, range 6 - 198 mg/l), and creatinine concentration was elevated in 18 (95%) patients (median maximum 321 μmol/l, range 74 - 1258 μmol/l).

Laboratory markers of coagulation and fibrinolysis

The values for coagulation variables during the acute phase and at recovery are shown in Table 1.

Table 1
Coagulation variables in the acute phase and at recovery in 19 patients with NE.

	Reference values	Acute phase	Recovery phase	p
APTT $_{max}$ (s)	23-33	34 (29-47)	29 (26-34)	<0.001
Prothrombin time $_{min}$ (%)	70-130	84 ± 23	119 ± 31	<0.001
Thrombin time $_{min}$ (s)	17-25	16 ± 1.6	19 ± 1.9	<0.001
Fibrinogen $_{min}$ (g/l)	1.7-4.0	3.6 ± 1.2	3.2 ± 0.8	0.14
F1 + 2 $_{max}$ (pmol/l)	69-229	726 (160-1461)	213 (95-351)	<0.001
D-dimer $_{max}$ (mg/l)	≤0.5	4.8 (0.3-19.5)	0.2 (0.1-1.4)	<0.001
Antithrombin activity $_{min}$ (%)	84-108	72 (52-113)	95 (64-119)	<0.001
Protein C activity $_{min}$ (%)	74-141	61 (32-121)	106 (64-185)	<0.001
Protein S free antigen $_{min}$ (%)	66-158	60 (19-90)	92 (68-121)	<0.001

Abbreviations: APTT = activated partial thromboplastin time, F1 + 2 = prothrombin fragments, max = maximum, min = minimum. The values represent mean or median and standard deviation or range (in brackets).

The most evident finding during the acute phase was markedly increased plasma D-dimer, the median maximum level being 24-fold higher than that measured at the control visit (Fig. 1a). D-dimer remained within normal values in only one patient. F1 + 2 concentration was 3.4-fold higher during the acute phase than at the control visit (Fig. 1b), and the concentration exceeded normal values in 18 of the 19 patients. D-dimer and F1 + 2 levels correlated positively ($r = 0.59$, $p = 0.007$). During the acute phase, coagulation activity (F1 + 2) appeared to correlate with the length of hospitalization ($r = 0.53$, $p = 0.02$). Neither D-dimer nor F1 + 2 correlated with creatinine concentration or platelet count (data not shown).

Acutely, the levels of natural anticoagulants PC and AT (Fig. 1c and d), as well as PS were low and TT was shortened ($p < 0.001$ for all) compared with the recovery phase. APTT was prolonged and PT% decreased ($p < 0.001$ for both) compared with the recovery values.

The reduced blood platelet count during the acute phase correlated with several variables. Especially strong correlations were observed between platelet count and AT and PC activities and PS ($r = 0.71$, $r = 0.73$, and $r = 0.81$, respectively, $p < 0.001$ for all). Correlation was also evident between platelet count and fibrinogen concentration ($r = 0.72$, $p < 0.001$).

A number of associations between CRP, AT, PC, PS, and fibrinogen were found, among the most distinct of them were the negative correlation between CRP and PC ($r = -0.58$, $p = 0.009$) and the positive correlation between PC and AT ($r = 0.73$, $p < 0.001$).

Renal function impairment, assessed by maximum creatinine concentration or minimum daily urinary output, did not correlate with the coagulation variables, except with the shortening of thrombin time ($r = -0.59$, $p = 0.007$ and $r = 0.53$, $p = 0.02$, respectively).

Five of the 19 patients (26%) reached at least five points in the ISTH score, indicating a diagnosis of DIC (Table 2). In addition to the typical low platelet count and high D-dimer, all five had prolonged APTT and elevated F1 + 2. One of the patients with DIC needed transient hemodialysis and experienced oozing at the site of the central venous catheter. However, there was no statistically significant difference between a positive DIC score and the length of hospitalization, the maximum creatinine concentration, the need of transient hemodialysis, or the bleeding tendency (data not shown).

Coagulation results of DIC negative and DIC positive patients according to the ISTH score are presented in Table 3.

Discussion

The findings of enhanced thrombin generation and fibrinolysis were conspicuous. An over 3-fold increase in F1 + 2 during the acute compared with the recovery phase indicated thrombin formation, and it applied to 18 of the 19 patients. Disproportionate to the thrombin generation, the 24-fold increase in D-dimer during the acute phase indicated markedly upregulated fibrin degradation and turnover or reduced elimination of the fibrin fragments. It could be attributable to the endothelial activation often suspected to be involved in the pathogenesis of acute NE [14,15]. However, the lack of overt coagulation

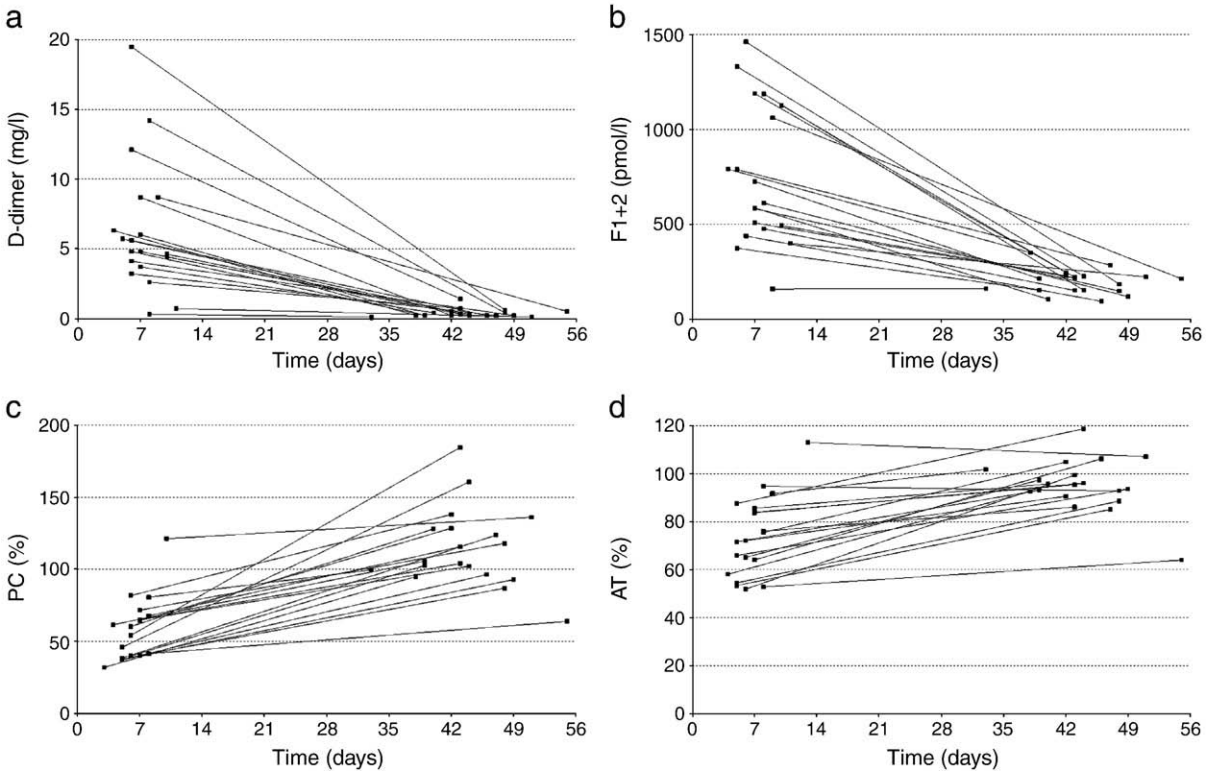

Fig. 1. Concentrations of (a) D-dimer and (b) prothrombin fragments (F1 + 2) and activities of (c) protein C (PC) and (d) antithrombin (AT) in 19 patients with NE. Values represent maximum (a,b) or minimum (c,d). The timing is counted from the beginning of the fever.

Table 2
Data of five patients compatible with DIC according to the ISTH score (5 or over).

Pt	Gender (F / M)	Age (years)	Dialysis	Platelet count min ($x10^9$/l)	PT min (%)	APTT max (s)	D-dimer max (mg/l)	Fibrinogen min (g/l)	F1 + 2 max (pmol/l)	DIC score
1	M	34	no	31	70	35	5.6	3.5	438	5
2	F	49	no	34	83	41	12.1	1.50	1125	5
3	M	30	no	39	40	38	19.5	1.8	726	6
4	M	36	no	36	76	46	6.0	2.4	1461	5
5	M	36	yes	13	60	40	3.2	2.5	789	5

Reference ranges: platelet count 150-360 × 10^9/l, PT% 70-130%, APTT 23-33 s, D-dimer ≤ 0.5 mg/l, fibrinogen 1.7-4.0 g/l, and F1 + 2 69-229 pmol/l. Abbreviations: min = minimum, max = maximum.

problems indicated a beneficial clinical balance between prothrombotic and fibrinolytic activities, despite the laboratory abnormalities. Interestingly, enhanced thrombin generation associated with the prolongation of hospital stay, one way of assessing the severity of the disease.

DIC promoted by vascular injury is considered common in HFRS and is often suggested as a possible cause of thrombocytopenia in NE [14]. However, the definition and laboratory measurements applied to diagnose DIC during NE vary greatly in the literature. Settergren et al reported 5% of 74 patients as having DIC, defined as clinically evident bleeding, positive ethanol gelation test, elevated levels of fibrin degradation products and platelets less than 100 × 10^9/l [12]. Four fatal cases of NE with assumed DIC from Finland and some sporadic cases elsewhere have been reported [8,11,16]. We evaluated our data with the uniform scoring system for DIC developed by the ISTH [13]. In addition to platelet count the score is based on PT and concentrations of D-dimer and fibrinogen. Five of the 19 patients here (26%) reached at least five points indicative of DIC. Interestingly, only two of them showed abnormal prothrombin time, and only one had clinical, albeit minor, problems with hemostasis. The positive score did not associate with elevated D-dimer or F1 + 2, the two most prominent findings in the study. Neither did the score associate with the clinical variables, and it failed to predict outcome in this group of acute NE patients.

Despite the lack of overt coagulation problems, our patients were clinically quite ill: three of the 19 (16%) needed transient hemodialysis compared with the 5% presented in the literature [4]. However, no association was observed between the degree of renal impairment and the depth of thrombocytopenia, although both are well-known clinical markers of NE. Neither minimum daily urinary output, maximum creatinine nor the need of hemodialysis correlated with indicators of thrombin formation and fibrinolysis or increased consumption of coagulation inhibitors, with the remarkable exception of thrombin time. Thrombocytopenia and short thrombin time could imply microvesiculation, i.e. membrane shedding with markedly enhanced coagulation activity, as reported in severe inflammation, complement activation and immune-mediated thrombocytopenia induced by heparin or hepatitis [17–19].

Table 3
Coagulation results of DIC negative and DIC positive patients according to the ISTH score.

	Reference values	DIC negative	DIC positive	*p*
Platelet count $_{min}$ (× 10^9/l)	150-360	88 (50-238)	34 (13-39)	0.001
APTT $_{max}$ (s)	23-33	33 (29-47)	40 (35-46)	0.009
Prothrombin time $_{min}$ (%)	70-130	86 (64-140)	70 (40-83)	0.02
Thrombin time $_{min}$ (s)	17-25	16 (13-19)	16 (15-18)	0.9
Fibrinogen $_{min}$ (g/l)	1.7-4.0	4.0 (2.7-6.7)	2.4 (1.5-3.5)	0.005
F1 + 2 $_{max}$ (pmol/l)	69-229	584 (160-1330)	789 (438-1461)	0.4
D-dimer $_{max}$ (mg/l)	≤0.5	4.7 (0.3-14.2)	5.6 (3.2-19.5)	0.2
Antithrombin activity $_{min}$ (%)	84-108	78 (53-113)	55 (52-68)	0.01
Protein C activity $_{min}$ (%)	74-141	65 (32-121)	40 (38-54)	0.03
Protein S free antigen $_{min}$ (%)	66-158	70 (48-90)	36 (19-44)	0.001

Abbreviations: APTT = activated partial thromboplastin time, F1 + 2 = prothrombin fragments, min = minimum, max = maximum. The values represent median (range).

In this study many of the altered coagulation variables reflect the inflammation during the acute viral disease. The diminished activities for the natural anticoagulants AT, PC and PS were marked, but were also rapidly reversed after the acute phase of the disease. The close correlation between protein C and antithrombin appears particularly interesting. It could reflect their consumption, the shedding and downregulation of the regulatory elements of coagulation activity. Also, the simultaneous loss of natural anticoagulants and platelets suggests temporal interactions between platelets, their activation and impaired natural anticoagulants, compatible with endothelial injury or activation. The interaction of platelets with endothelium, their activation and P-selectin expression could provide mechanisms of thrombocytopenia during hantavirus infection, as in several other forms of viral inflammation [20–23].

Capillary leak and increased vascular permeability, as well as decreased platelet count are cardinal clinical features in both HFRS and HCPS, but the mechanisms leading to them are not fully understood [24]. Interestingly, pathogenic hantaviruses, which cause either HFRS or HCPS, use beta 3 integrins for entry to host cells [25]. Beta 3 integrins are abundant surface receptors on both endothelial cells and platelets, and regulate both vascular permeability and platelet activation and adhesion. The use of these receptors by hantaviruses may therefore be fundamental to hantavirus pathogenesis, and specificly to hantavirus-associated thrombocytopenia. Recently, the intensity level of platelet beta 3 integrin in patients with HFRS was suggested to be associated with disease severity [26]. Moreover, another newly discovered mechanism which associates with disease severity of HFRS is human platelet alloantigen (HPA) -3 polymorphism [27].

The earlier literature indicates hemostasis impairment in NE due to remaining dysfunctional platelets. This could result from acute renal failure and increased fibrin degradation, both confirmed in our study. Furthermore, exhausted circulating platelets could be implied, referring to their *in vivo* activation leading to impaired aggregation and release reactions when tested *in vitro* [14,28].

We are aware that the timing of the samples during the acute phase overlaps because the patients sought medical assistance at different time intervals from the beginning of their disease. Another limitation of the study is the relatively small number of patients included, which undermines the value of subgroups of the patients. However, this is the first study on coagulation and fibrinolysis activity during the acute phase and recovery of Puumala hantavirus infection. Our findings show that severe NE influences the platelets and the coagulation system and may sometimes result in DIC. In this study the altered coagulation was not important for the major outcome of the disease, namely, kidney function.

In conclusion, the conspicuous elevations of prothrombin fragments and especially D-dimer found in our study indicate increased thrombin formation and marked fibrinolytic activity during the acute phase of NE. Enhanced fibrinolysis could compensate for the coagulation activity and contribute to clinical recovery. In addition to the markers of endothelial involvement and the transient organ failure in the kidneys, further studies could focus on the functions of platelets, including microvesiculation, complement activation, and their interactions with coagulation and fibrinolysis [17,29,30].

Conflict of interest statement

The authors have no conflict of interest.

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Platelet ligands and ADAMTS13 during *Puumala hantavirus* infection and associated thrombocytopenia

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We aimed here to elucidate the role of adhesive platelet ligands and endothelial involvement during the acute phase of *Puumala hantavirus* (PUUV) infection. Nineteen hospital-treated patients with serologically confirmed diagnosis of acute PUUV infection were included. Patient charts were reviewed for clinical and basic laboratory data. Plasma levels of von Willebrand factor antigen (VWF : Ag), ristocetin cofactor (VWF : RCo), factor VIII (FVIII : C) and a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13 (ADAMTS13) activities as well as fibrinogen and fibronectin were measured three times acutely and once during the recovery phase. VWF : Ag and VWF : RCo were nearly three-fold higher acutely compared with recovery (median 252 vs. 88%, and mean 267 vs. 98%, respectively; $P < 0.001$ for both), whereas FVIII : C was only slightly elevated (median 118 vs. 88%, $P = 0.002$) and remarkably failed to show association with VWF in the acute phase. ADAMTS13 activity and fibronectin concentration were lower in the acute compared with the recovery phase (median 56 vs. 63%, $P = 0.003$, and median 221 vs. 330 μmol/l, $P = 0.001$, respectively). Fibrinogen raised acutely (mean 5.0 vs. 3.3 g/l, $P < 0.001$), negatively correlating with the platelet count ($r = -0.468$, $P = 0.043$). Markedly upregulated fibrinogen and VWF together with decreased levels of ADAMTS13 activity and fibronectin were observed during acute PUUV infection. VWF and FVIII : C did not associate during the acute phase, whereas thrombocytopenia correlated negatively with fibrinogen. These findings imply several rearranged interactions between platelets and their ligands. *Blood Coagul Fibrinolysis* 22:468–472 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Puumala hantavirus infection (PUUV), also known as nephropathia epidemica, is the most common cause of hemorrhagic fever with renal syndrome (HFRS) in Europe, some 1000–2000 serological diagnoses being made annually in Finland [1,2]. The acute illness is characterized by high fever, headache, back and abdominal pains, visual disturbances, hemorrhages and acute impairment of renal function. The clinical severity of acute PUUV infection varies from mostly mild, even subclinical disease to rarely reported fatal cases. Thrombocytopenia is encountered almost invariably in nephropathia epidemica but bleeding manifestations are usually mild [3,4].

The cellular entry of hantaviruses which cause HFRS is mediated by β_3 integrins expressed on platelets, endothelial cells and macrophages [5]. Pro-inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor α, and interleukin-1 (IL-1) are released and have a modifying effect on vascular permeability. The increased capillary permeability manifested by vascular leakage explains many symptoms and features of nephropathia epidemica [6,7]. Enhanced cytokine activity also increases expression of intercellular and vascular cell adhesion molecules, as has been detected in kidney samples obtained during the acute phase of nephropathia epidemica [8,9]. The increased expression of adhesion molecules is likely to associate with procoagulant changes in platelets and endothelium during the acute phase [7]. Recently, we reported enhanced thrombin formation and fibrinolysis during the acute phase of nephropathia epidemica [10].

Here we assessed adhesive platelet ligands related to endothelial function and acute-phase reactions in the early phase of nephropathia epidemica and at recovery. We sought to elucidate the role of these adhesion molecules in the pathogenesis of nephropathia epidemica and to establish possible associations between laboratory findings and the clinical course of the disease.

Methods

The collection of clinical data including the routine laboratory measurements as well as the collection of study blood samples and processing to plasma as described in detail in our recent article [10]. The study

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included 19 hospitalized patients with serologically confirmed acute PUUV infection (17 men, median age of patients 38 years, ranging from 30 to 64 years). The fibronectin samples were collected in EDTA, whereas other samples were collected in 3.2% sodium citrate. During the acute phase of the disease three samples were obtained. The first was collected on admission as early as possible, 2–9 (median 6) days after the onset of fever. The second was obtained 3–10 (median 7) days and the third 6–13 (median 10) days after the onset of fever. The last sample in the study, that is the control sample, was collected at full recovery (ranging 32–54 days from fever onset, median 43 days).

The activity of a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13 (ADAMTS13) was determined by immunochemical detection of its proteolytic target, that is von Willebrand factor (VWF; Technozym Elisa, Technoclone, Vienna, Austria). Factor VIII activity (FVIII : C) was determined by one-stage clotting assay (Pathromtin SL and Coagulation Factor VIII Deficient Plasma; Siemens Healthcare Diagnostics, Marburg, Germany). Von Willebrand factor antigen (VWF : Ag) and ristocetin cofactor activity (VWF : RCo) were measured with the BCS XP analyzer with BC von Willebrand Reagent and VWF : Ag Latex Reagent (Siemens Healthcare Diagnostics). Fibrinogen was measured with a modification of the Clauss method (Multifibren U; Siemens Healthcare Diagnostics). Fibronectin concentration was determined at room temperature by enzyme-linked immunosorbent assay (human Fibronectin ELISA; Bender MedSystems, Vienna, Austria).

Plasma IL-6 was measured on three consecutive mornings starting the first morning of hospital stay. The concentration was determined using a commercially available enzyme-linked immunosorbent assay (PeliKine Compact human IL-6 kit; Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). The detection limit for the assay was 0.4 pg/ml.

The study was carried out in Tampere University Hospital and Helsinki University Central Hospital, Finland and was approved by the Ethics Committees of these hospitals.

Statistical analysis

The highest value of continuous variables known to increase during the infection was chosen to represent the maximum for the variable in the acute phase. The lowest of three ADAMTS13 and fibronectin values and platelet counts were taken to represent the minimum values. Paired samples *t*-test or Wilcoxon's test was used when appropriate. Pearson's or the Spearman rank correlation coefficient was calculated. Comparisons were made using the Mann–Whitney *U*-test. The limit of significance was set at 0.05 (two-tailed). SPSS 7.5 (SPSS Inc., Chicago, Illinois, USA) was used for computation.

Results

During the acute disease phase thrombocytopenia was found in 15 of the 19 patients, but by the time of discharge, 17 had platelet counts within normal values. The median lowest daily urinary output was 880 ml, and three of the 19 patients needed transient hemodialysis treatment. The acute IL-6 concentration was 16.7 pg/ml (median, range 3.6–96.6). Four patients with minor bleeding symptoms had higher IL-6 levels, leukocyte counts and C-reactive protein values than those without ($P = 0.007$, $P = 0.019$ for the last two, respectively).

VWF : Ag and VWF : RCo levels were nearly three-fold higher during the acute compared with the control phase (Table 1). Eighteen patients evinced elevated VWF : Ag levels (Fig. 1a), in contrast we noted only a slight increase in FVIII : C acutely. VWF : Ag and VWF : RCo levels correlated well with each other ($r = 0.94$, $P < 0.001$), but intriguingly not with FVIII : C. The ratio between VWF : Ag and FVIII : C was nearly three-fold higher acutely compared with the control phase (median 3.72 vs. 1.25, $P < 0.001$). FVIII : C correlated with fibrinogen ($r = 0.589$, $P = 0.008$). Fibrinogen was 1.6-fold higher during the acute compared with the control phase, and 14 patients had acutely elevated fibrinogen levels (Fig. 1b). The negative correlation prevailed between fibrinogen and platelet count ($r = -0.468$, $P = 0.043$).

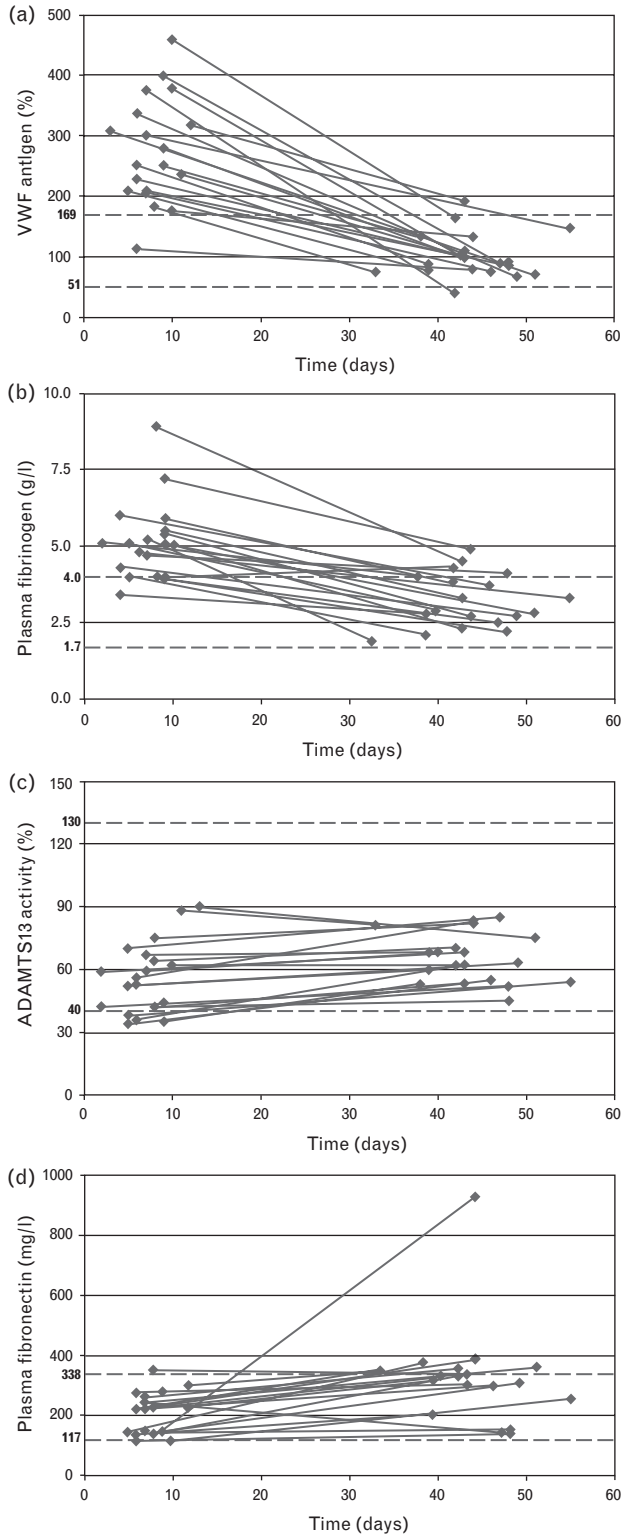
ADAMTS13 activity was lower in the acute phase compared with the control phase (Fig. 1c). However, only four patients had their minimum values below the lower reference limit.

Table 1 Laboratory markers of coagulation and endothelial cell activation during the acute phase and at recovery in 19 patients with nephropathia epidemica

Variable	Reference values	Acute phase	Recovery phase	*P*
VWF : Ag (%)	51–169	252 (113–459)	88 (39–192)	<0.001
VWF : RCo (%)	44–183	267 ± 112	98 ± 39	<0.001
FVIII : C (%)	52–148	118 (56–173)	88 (39–192)	0.002
Fibrinogen (g/l)	1.7–4.0	5.0 ± 1.3	3.2 ± 0.9	<0.001
ADAMTS13 activity (%)	40–130	56 (34–90)	63 (45–85)	0.003
Fibronectin (μg/ml)	117–338	221 (114–350)	330 (138–928)	0.001

Values represent means (±SD) or medians (range). ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 domain 13; FVIII : C, factor VIII activity; VWF : Ag, von Willebrand factor antigen; VWF : RCo, VWF ristocetin cofactor activity.

Fig. 1



Von Willebrand factor antigen (VWF : Ag) (a), fibrinogen (b), ADAMTS13 activity (c), and fibronectin (d) during the acute phase and at recovery in 19 patients with *Puumala hantavirus* infection. The reference ranges are indicated by broken lines. Days are counted from the onset of fever.

Fibronectin was also low in the acute phase (Fig. 1d), the mean being 1.6-fold higher in the control phase. Only two patients had minimum values below the lower reference limit.

Discussion

We here assessed the role of platelet ligands VWF, fibrinogen and fibronectin in the acute phase of nephropathia epidemica and related the findings to the clinical course of the disease. In addition to mediating platelet–platelet interaction, the adhesion molecules in question are carried by platelets and implicated in acute-phase reaction and endothelial activation. We found an almost three-fold increase in the levels of plasma VWF : Ag and VWF : RCo and a 1.6-fold increase in fibrinogen in the acute phase of nephropathia epidemica compared with the control phase.

VWF is produced by the endothelium and megakaryocytes and carried in the α-granules of circulating platelets. VWF enhances early platelet adhesion and aggregation to injured endothelium during both physiological primary hemostasis and pathological thrombosis [11]. Aside fibrinogen and fibronectin it is an important ligand of $\alpha IIb\beta_3$ which contains the β_3 integrin receptor for hantaviruses capable of causing HFRS [5,12]. An increased VWF level could imply endothelial cell injury and exposure of the adhesive matrix to platelets. Also, strong platelet activation and granule release could increase circulating VWF levels, which in this study raised three-fold. These mechanisms of enhanced platelet adhesion and activation could result in platelet consumption and the thrombocytopenia encountered in the acute phase of nephropathia epidemica, as described in dengue virus infection [13,14].

Fibrinogen, another major platelet constituent and external ligand of $\alpha IIb\beta_3$, contributes to thrombus stability by anchoring the thrombi to the vessel wall and individual platelets to the thrombus [12]. The fibrinogen concentration in plasma increased 1.6-fold acutely compared with the control phase. This could imply platelet activation and release of fibrinogen as well as inflammation-triggered synthesis of fibrinogen by the liver. Elevated fibrinogen could reflect an acute-phase reaction in the liver strong enough to outweigh the consumption due to the coagulation activity ongoing during the acute phase of nephropathia epidemica, as observed in our previous study [10]. Such a conception is supported by the negative correlation between fibrinogen and platelet count, as a decreasing platelet count may mirror increased platelet activation and consumption. The finding of increased VWF and fibrinogen may counterbalance bleeding tendency, despite the low platelet count encountered in the acute phase of the disease.

Fibronectin, the third ligand of $\alpha IIb\beta_3$, is a high-molecular-weight glycoprotein present in a soluble form

in plasma and other body fluids and in insoluble form in the extracellular matrix [12,15]. Fibronectin is firmly bound to fibrinogen and fibrin at low temperatures *in vitro* (at 0–4°C) and subsequently released as the temperature rises [16]. Fibronectin participates in plug formation by gluing platelets to each other, thus enhancing thrombus formation, especially under arterial share rates, such as in the microcirculation [17]. Recently, increased serum fibronectin levels in patients with HFRS caused by Hantaan virus were reported to be associated with disease severity [18]. However, the temperature at which the ELISA analyses were carried out was not provided. Another group has previously reported diminished fibronectin levels as a marker of sepsis [19]. We observed a considerable decrease in plasma fibronectin concentrations in the acute phase of nephropathia epidemica. The binding of fibronectin to platelets is enhanced by thrombin but not by other platelet activators [20]. This may be compatible with our previous finding of thrombin activity in early hantavirus infection [11], and a loss of fibronectin could reflect the binding to thrombin-activated platelets and consumption during coagulation activity.

In circulation FVIII is bound to VWF while inactive and degrades rapidly when released from VWF by the action of thrombin [11]. Interestingly, in our study the increase in FVIII : C did not follow VWF levels and the ratios between VWF : Ag and FVIII in the acute and control phase markedly differed. This may be in keeping with our recent observation of enhanced thrombin generation during the acute phase of nephropathia epidemica and utilization of FVIII in coagulation and fibrin formation [10]. FVIII may also be bound to microparticles from platelets stimulated by thrombin or the complement proteins C5b-9 [21,22]. The finding of only modest increase in FVIII : C is also compatible with dengue virus infection, in which reduced coagulation factors correlated with the degree of thrombocytopenia and the clinical severity of the disease [23].

A decrease in the activity of ADAMTS13, an enzyme specifically involved in the cleavage of high-molecular-weight VWF, is known to have an important role in the pathogenesis of thrombotic thrombocytopenic purpura (TTP). This form of thrombotic microangiopathy is characterized by thrombocytopenia, hemolytic anemia, and ischemic organ failure due to thrombotic occlusions in the arterioles [24]. Thrombocytopenia is frequent in acute nephropathia epidemica and variable neurological symptoms (headache, visual disturbances, even acute disseminated encephalomyelitis and Guillain–Barré syndrome) are also encountered [25]. In our study, ADAMTS13 activity was modestly reduced during the acute phase of nephropathia epidemica, in accordance with studies implying that decreased ADAMTS13 activity is not restricted to TTP [14,26].

The participants here evinced quite severe organ failure in the kidneys. Three of the 19 patients needed transient hemodialysis compared with the 5% presented in the literature [2]. In this small study, the degree of renal impairment was not associated with any of the platelet ligands or fibronectin. Low platelet count was predicted by the high level of fibrinogen.

In conclusion, markedly enhanced VWF with only a slight increase in FVIII together with decreased levels of ADAMTS13 activity and plasma fibronectin were observed during the acute phase of nephropathia epidemica. The findings support previous evidence of the role of activated platelets and the endothelium in the pathogenesis of nephropathia epidemica. Further studies are warranted on platelet activation and the formation of microparticles during the acute phase of nephropathia epidemica.

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Conflicts of interest

There are no conflicts of interest.

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Complement activation in Puumala hantavirus infection correlates with disease severity

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Running title: Complement activation in hantavirus infection

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Abstract

Introduction: Hantaviruses are important human pathogens that cause clinical diseases characterized by renal and cardiopulmonary manifestations. Their pathogenesis is currently poorly understood. We have studied the role of the complement system in the pathogenesis of Puumala (PUUV) hantavirus infection.

Material and Methods: We studied the activation of complement by measuring the terminal complement complex SC5b-9 and complement component C3 and C4 levels in patients with acute PUUV infection. Several laboratory parameters and clinical findings reflecting the severity of PUUV-HFRS were evaluated with regard to complement activation.

Results: The levels of SC5b-9 were significantly increased and C3 decreased in the acute stage as compared to the levels at full recovery ($P<0.001$). We found that SC5b-9 levels were higher in patients with chest x-ray abnormalities than in patients with a normal x-ray during the acute stage ($P=0.028$). Furthermore, SC5b-9 and C3 levels showed significant correlation with several clinical and laboratory parameters that reflect the severity of the acute PUUV infection.

Conclusions: We showed that complement system becomes activated via the alternative pathway in the acute stage of PUUV infection and the level of activation correlates with disease severity. The results further suggest that complement activation may contribute to the pathogenesis of acute PUUV infection.

Keywords: C3, C4, complement, hantavirus, HFRS, SC5b-9

Key messages:

1) Complement activation via the alternative pathway in the acute stage of PUUV hantavirus infection correlates with disease severity.

2) Complement activation may contribute to the pathogenesis of acute PUUV infection.

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Abbreviations

HFRS- hemorrhagic fever with renal syndrome

HCPS- hantavirus cardiopulmonary syndrome

NE- nephropathia epidemica

MAC-membrane attack complex

PUUV- Puumala virus

Introduction

Puumala virus (PUUV), a member of the *Hantavirus* genus in the Bunyaviridae family, is a rodent-borne zoonotic virus with a segmented, negative-stranded RNA genome. PUUV is carried by the bank vole, *Myodes glareolus,* which is found in most of Europe [1]. Numerous hantaviruses are known pathogens to humans and cause two diseases: hemorrhagic fever with renal syndrome (HFRS) in Eurasia, and hantavirus cardiopulmonary syndrome (HCPS) in the Americas (1). Acute PUUV infection, known as nephropathia epidemica (NE), is a mild form of HFRS with approximately 0.1% mortality (2).

PUUV-HFRS has an incubation period of 2-6 weeks and the disease begins with high fever, headache, nausea and vomiting. Signs of renal insufficiency include proteinuria, hematuria and increased creatinine levels as well as oliguria followed by polyuria. Transient hemodialysis treatment is needed in approximately 5% of hospitalized patients (3). General laboratory findings include leukocytosis, thrombocytopenia, hypoproteinemia and increased CRP levels. The severity of the disease is associated with HLA B8, DR3 and DQ2 haplotype (4).

Pulmonary involvement has been described in about one third of PUUV-infected patients (5-8) and over half of the patients have abnormal cardiac findings (9). It is believed that capillary leakage due to increased capillary permeability plays a role in the pathogenesis of pulmonary changes in HFRS and HCPS, which is more frequently characterized by severe pulmonary dysfunction (10,11).

A previous study on complement activation in PUUV infection showed that complement activation is common and suggested that the classical pathway of complement is associated with disease severity (12) . However, the role of the complement system in the pathogenesis of PUUV-HFRS has not been further studied in larger patient populations. The complement

system has three major pathways; the classical, alternative and the lectin-dependent pathway. These pathways are activated differently but they all converge on complement component C3 that has a key function in the complement system (13). The end product of the complement cascade is the cytolytic membrane attack complex (MAC), formed by sequential assembly of complement proteins C5b, C6, C7, C8 and C9 to a target cell membrane. When the complexes are formed in the absence of a target membrane in the fluid phase, C5b-9 binds to S-protein (vitronectin) or clusterin and a non-lytic soluble SC5b-9 terminal complex is formed (14).

We measured the levels of SC5b-9, C3 and C4 in 61 hospitalized patients in the acute stage of PUUV infection and at recovery to study the role of complement in the pathogenesis of PUUV-HFRS.

Material and Methods

Ethics statement

Written informed consent was obtained from all patients, and the Ethics Committee of the Tampere University Hospital approved the study protocol.

Patients and methods

This prospective study included 61 consecutive hospitalized patients with serologically confirmed acute PUUV infection. The serological diagnosis is based on μ-capture PUUV-IgM enzyme immunoassay (15). All patients, 44 males and 17 females, median age 46 (range; 22-77), were treated at the Tampere University Hospital during the period from September 2000 to January 2004.

The median number of plasma samples taken from patients during hospital care was 4 (range 1-6). The first sample (n=61) was obtained upon admission to hospital 2-16 (median 5) days after the onset of fever. The second sample (n= 58) was obtained 4-16 (median 6), the third sample (n=45) 6-17 (median 8), the fourth sample (n=31) 6-14 (median 9), the fifth sample (n=10) 7-11 (median 10) and the sixth sample (n=2) 13-18 (median 16) days after the onset of fever. The last sample (n=53) was taken at full recovery 18-55 days (median 38) after the onset of fever and represented the control sample in the analyses.

Laboratory parameters, determined at the Laboratory Center of the Tampere University Hospital, and clinical findings were retrieved from patient charts. All chest radiographs were studied retrospectively by a radiologist (A.P.). Complement analyses were performed at the Haartman Institute and at HUSLAB. Plasma C3 and C4 levels were measured by nephelometry (Dade Behring, Marburg, Germany) and SC5b-9 using an ELISA kit (Quidel, San Diego, CA). Different sets of samples were used for SC5b-9 and C3/C4 analyses and the

samples were stored at -70 °C or -20°C, respectively. A number of patient samples did not meet the quality-control criteria for the measurements of C3, C4 and SC5b-9 levels defined by the manufacturer. Thus, these samples were excluded from the respective analyses.

Statistical analyses

Statistical analyses were performed using SPSS software version 18 (USA). The highest and lowest values of continuous variables measured during hospital care were designated as maximum or minimum values. As for the SC5b-9 the maximum, and for the C3 and C4 levels, the minimum values during the acute stage, respectively, were used. The maximum level of SC5b-9 reflects the peak of complement activation and minimum level of C3 and C4 the degree of C3 and C4 consumption during the acute stage of PUUV infection.

We used Mann-Whitney U test to compare SC5b-9, C3 and C4 levels between different patient groups and Wilcoxon's rank sum test to compare the levels in the same individual at different time points (acute stage and full recovery). To express a relationship between the continuous variables, Spearman's rank correlation coefficients were computed. All P-values are two tailed and statistical significance was considered at 5% level.

Results

SC5b-9, C3 and C4 levels. We measured the plasma levels of SC5b-9, C3 and C4 during the hospital care and at full recovery. The highest and lowest concentrations of SC5b-9 and C3, respectively, were found in the first or second acute-stage sample in nearly all patients (n=50) and from the third or fourth sample in the others. The lowest concentration of C4 was found in the first or second acute-stage sample in 40 patients.

As shown in Figure 1A, the median maximum plasma SC5b-9 concentration in the acute stage was significantly higher than at full recovery (493 ng/ml, range 103- 1034 ng/ml vs. 197 ng/ml, range 100- 522 ng/ml, $p<0.001$). The acute-stage median minimum C3 level was decreased compared to the level measured at full recovery (1.26 g/l, range 0.65-2.24 g/l vs. 1.47 g/l, range 0.84-2.44 g/l, $p<0.001$, Figure 1B). The acute-stage median minimum C4 level was not significantly different from the level measured at full recovery (0.26 g/l, range 0.08-0.52 g/l vs. 0.27 g/l, range 0.09-1.1 g/l, $p=0.48$, Figure 1C).

Fifty patients had chest x-ray taken upon hospitalization. Fourteen of them (28%) had abnormal findings including accumulation of pleural fluid (n=6) and atelectasis (n=12). The patients with chest x-ray abnormalities during the acute stage had significantly higher levels of SC5b-9 than patients with normal x-ray ($P=0.028$) but the levels were no longer different at the time of recovery (Figure 2A). The patients with pathological findings in chest x-ray in the acute stage tended to have lower minimum C3 levels than patients with normal x-ray but the difference was not statistically significant ($P=0.08$) (Figure 2B). No differences were observed in C4 levels (Figure 2C).

Correlation of SC5b-9, C3 and C4 levels with disease severity. Clinical and laboratory findings of patients are shown in Table 1. Acute-stage levels of SC5b-9 and C3 correlated significantly with a number of variables that reflect the clinical severity of PUUV infection

(Table 2). As for maximum levels of SC5b-9, the strongest correlations were found with the treatment time at hospital, the highest blood leukocyte count, and the change in weight during hospital care ($r=0.46$, $r=0.43$ and $r=0.42$, respectively, $P<0.001$ for all). The variables that showed the strongest correlation with minimum C3 levels were the highest levels of IL-6, the lowest blood hematocrit level, and lowest serum sodium level ($r=-0.62$, $r=0.52$ and $r=0.44$, respectively, $P<0.001$ for all). The highest blood leukocyte count also correlated inversely with C3 levels ($r=-0.3$, $P=0.020$). Acute-stage levels of C4 did not show significant correlation with any of the clinical or laboratory findings (Table 2).

Discussion

Our study shows that the alternative pathway of complement system becomes activated in the acute stage of PUUV infection, observed as elevated levels of SC5b-9 and decreased levels of C3 (Figure 1). The levels of C4 were not significantly altered between the acute stage and full recovery. The actual peak of complement activation, however, had possibly occurred before the patients were hospitalized. Furthermore, we showed that SC5b-9 levels were significantly higher in patients with chest x-ray abnormalities than in patients with a normal x-ray during the acute stage of PUUV-HFRS (Figure 2A). We also found that consumption of C3 was higher among patients with x-ray abnormalities, although this was not statistically significant (Figure 2B), possibly due to the small sample size. In general, we consider the level of SC5b-9 a better measure of complement activation since C3 levels are influenced not only by consumption but also by increased synthesis related to the acute phase response. This likely explains why some individuals had higher C3 levels in the acute stage of PUUV-HFRS than at the recovery (Figure 1B).

Previous studies have suggested that the pathogenesis of lung disease in HCPS and HFRS is attributable to increased capillary permeability in lungs (11). Puumala and Sin Nombre hantaviruses can infect endothelial cells in vitro without causing any visible changes in cell morphology or necrosis in the infected endothelium in vivo (10,16). Therefore, it is believed that immunological mechanisms contribute to changes disturbing the function of endothelium and thereby lead to capillary leakage. It has been suggested that cytotoxic CD8+ T cells would trigger capillary leakage (17) and that cytokines may contribute to the increased capillary permeability (5,18).

Studies in the rat have shown that the soluble form of C5b-9 (SC5b-9) can increase the endothelial permeability by ligating $\alpha_v\beta_3$-integrin of lung endothelium by increasing the

hydraulic conductivity in a dose-dependent manner (19,20). SC5b-9 can also promote the permeability in human endothelial cells through the release of bradykinin and platelet activating factor (21) and SC5b-9 has been shown to promote pulmonary edema in the adult respiratory distress syndrome (ARDS) (22). Based on our findings, it is possible that complement activation during the acute stage of PUUV-HFRS may contribute to the pathogenesis of vascular leakage in the lungs. Although pleural fluid indicating leakage was not visible in all patients with pathological findings in the chest x-ray, the presence of fluid cannot be excluded since small amount of fluid accumulation is not detectable in posteroanterior radiographs (23). Our data cannot, however, prove a direct causal link between complement activation and vascular leakage since no direct experimental evidence is provided. Additional immunopathological mechanisms could be involved.

The role of complement system in the pathogenesis of other viral diseases has recently been studied. Increased permeability of the endothelium without morphological damage to the cells is a key feature of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (24) and recent studies showed that patients with DHF had significantly higher SC5b-9 and lower C3 levels in plasma than patients with less severe dengue fever during the acute stage of the disease (25,26). The authors observed highest levels of SC5b-9 in the pleural fluids and plasma of DSS patients (25). The SC5b-9 levels found in our study were however clearly lower than in DSS patients, which is in line with the milder course of PUUV infection compared to DSS. However, our levels were similar to those seen in DHF patients.A recent study also suggested that complement activation contributes to a more severe clinical outcome of H1N1 pandemic influenza (27) .

We showed in our study that complement system activation, observed as elevated levels of SC5b-9 and/or lower levels of C3, in the acute stage of PUUV-HFRS correlated significantly with several laboratory and clinical findings that reflect the disease severity such as

leukocytosis and thrombocytopenia (Table 2). One previous study showed that low serum protein level, a good marker of capillary leakage, was strongly associated with abnormal chest x-ray findings (7). However, we did not have protein levels available in this study. The length of hospital treatment, probably the most objective measure to define severity, showed significant correlation with both high SC5b-9 and low C3 concentrations.

We observed an inverse correlation between the C3 and IL-6 levels in the acute stage of PUUV infection (Table 2). Significant increase in plasma IL-6 levels has been reported in patients with acute PUUV infection (28) and IL-6 secretion has been found to stimulate C3 synthesis (29). In our study, the low C3 levels associated with high IL-6 levels probably reflect the level of tissue injury and consumption of C3 in the acute stage of the disease. An increase in C3 levels due to IL-6 secretion was probably not observed at the time samples were taken because it usually follows a 2-5 days delay.

Our hypothesis is supported by our recent findings concerning two fatal cases of PUUV-HFRS (unpublished data). The first patient with severe pulmonary edema had high SC5b-9 and low C3 levels during the acute stage of PUUV infection as compared to the levels found in this study and the immunohistochemical staining showed extensive accumulation of C5b-9 and C3 in lung tissue. Heavy deposition of C5b-9 and C3 complexes was also detected in hypophysis, characterized by hemorrhages, and in the liver. As for the second patient, plasma levels of complement proteins were not obtained but immuhistochemical staining showed similar accumulation, although to a slightly lesser extent, of C5b-9 and C3 in lungs and hypophysis.

We measured the level of SC5b-9 that reflects overall activation of the complement system in the specimen. SC5b-9 is the soluble equivalent of the membrane-associated MAC complex. Therefore, the result indirectly suggests that formation of membrane attack complexes,

known to trigger cellular reactions and production of inflammatory cytokines (30), also contributes to the pathology of PUUV-HFRS. In addition to the terminal complement complex, elevated plasma levels of C5a, known to play role in the pathogenesis of ARDS (31), may also contribute to pulmonary dysfunction in PUUV HFRS as C5a attracts and aggregates leukocytes that can result in capillary obstruction and leakage (29). Correlation between complement activation and blood leukocyte count was observed in our study.

The sample size of this study was relatively small. Thus, it has an impact on the statistical power and addresses the need for further studies. However, for a prospective panel of hantavirus patient samples studied comprehensively with similar methodology, the number of cases is considerable. The time elapsed from the onset of fever to the time point when the first acute-stage samples were taken varied between individual patients. This time frame was not, however, significantly different between the patients with chest x-ray abnormalities and other patients (data not shown). Thus, analyses on SC5b-9 and C3 levels were presumably not biased. There were no significant differences in the age and sex distribution or in the frequency of previous illnesses between the patients with chest x-ray abnormalities and other patients.

The only previous study on complement activation in PUUV infection (12) indicated that complement activation via the classical pathway is associated with the disease severity. Our study, with a considerably larger sample size, confirmed that complement becomes activated in the acute stage of PUUV and further extends the findings by demonstrating that SC5b-9 and C3 levels, indicating the alternative pathway activation, correlated with disease severity. Although our data showed that C4 levels were not significantly altered between the acute stage and recovery, it cannot be excluded that classical pathway activation also occur as it is possible that consumption is compensated by increased synthesis. However, our data

indicated that the activation of the classical pathway is not as strong or long-standing that it would be observable in changes in total C4- levels.

Our study did not address the question of what actually triggers the complement activation in PUUV-HFRS and why do some individuals have stronger responses than others. A previous study showed that individuals with the HLA B8, DR3 and DQ2 haplotype are likely to have severe PUUV-HFRS (4), and a more recent study established that the presence and the severity of abnormal chest radiography findings are apparently associated with this haplotype (6). This haplotype regularly carries a deletion of the C4A gene that encodes the C4A protein, one of the two isotypes of complement component C4. C4 is an essential component of the complement system and deficiencies of C4A are associated with defective clearance of immune aggregates and persistence of viral infections (32). Abnormal complement activation may thus be associated with this particular HLA haplotype and mechanistically, it may relate to the deficiency of C4A. The C4A deficiency may lead to impaired classical pathway activation and an inability to process immune aggregates, e.g. antigen-antibody complexes, which in turn would lead to further inflammation and complement activation via the alternative pathway. However, the total plasma levels of C4 measured in this study does not indicate the level of C4A proteins specifically. Individuals with elevated complement activation may also have impaired regulation of the alternative complement pathway. A recent study showed that alternative complement pathway deregulation correlates with the severity of DHF and this may be linked to complement factor H dysfunction (26).

Taken together, our data indicate that complement activation via the alternative pathway in the acute stage of PUUV hantavirus infection correlates with disease severity and may contribute to the pathology of PUUV-HFRS. The complement system may thus be considered a potential therapeutic target to suppress severe hantavirus disease.

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Tables

Table 1. Clinical and laboratory findings in 61 patients with acute PUUV-HFRS

	Median (Range)
Clinical finding:	
$SBP_{min,}$ *mmHg*	110 (82-162)
$DBP_{min,}$ *mm Hg*	70 (46-100)
$Diur_{min,}$ *ml*	1620 (50-5800)
$Diur_{max,}$ *ml*	3780 (1280-9740)
Change in weight, *kg*	2.7 (0-12)
Treatment time at hospital, *days*	6 (2-15)
Laboratory finding**:**	
Leukocyte $count_{max,}$ $10^9/l$	9.9 (3.9-31.2)
$Hcr_{min,}$ *%*	0.36 (0.25-0.43)
$Hcr_{max,}$ *%*	0.44 (0.34-0.57)
Platelet $count_{min}$ $10^9/l$	67.5 (9-238)
S-$Crea_{max}$ µmol/l	175 (65-1285)
IL-$6_{max,}$ *pg/ml*	11.6 (1.3-96)
S-$Na_{min,}$ *mmol/l*	131 (115-139)

NOTE. $SBP_{min=}$ lowest systolic blood pressure, DBP_{min} = lowest diastolic blood pressure, $Diur_{min=}$ lowest daily urinary output, $Diur_{max}$= highest daily urinary output, Leukocyte $count_{max}$ =highest blood leukocyte count (normal range 3.4-8.2 x 10^9/l), Hcr_{min}= lowest blood hematocrit (normal range for women 35-46%, and for men 39-50%), Hcr_{max}= highest blood hematocrit, Platelet $count_{min}$= lowest blood platelet count (normal range 150-360 x 10^9/l), S-$Crea_{max}$= highest serum creatinine concentration (normal range for women 50-90 µmol/l, and for men 60-100 µmol/l), IL-6_{max}=highest serum interleukin-6 level, S-Na_{min}= lowest serum sodium level (normal range 137-145 mmol/l).

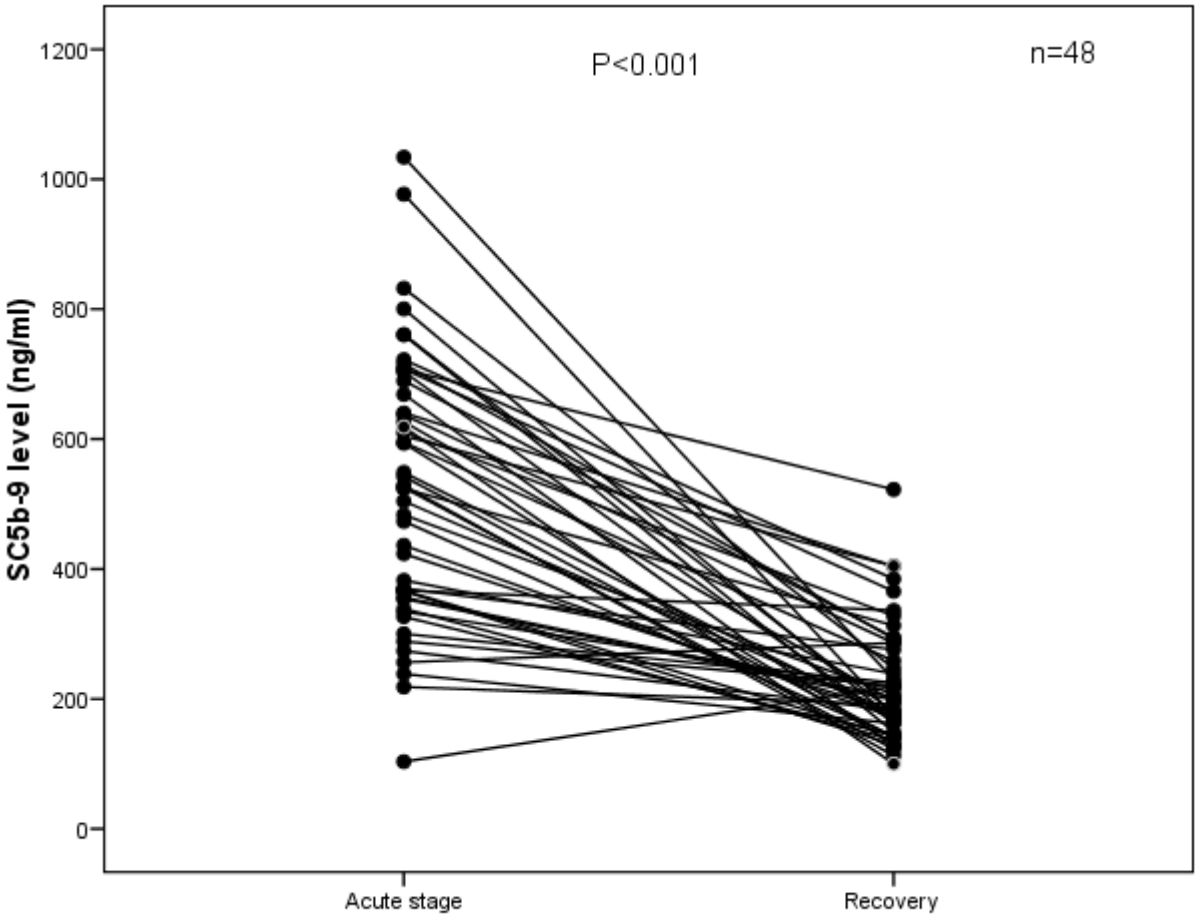
445 **Table 2.** Spearman rank correlations of minimum acute-stage level of C3 and C4 and
446 maximum acute-stage level of SC5b-9 with clinical and laboratory findings in PUUV-HFRS
447 patients

	SC5b-9		C3		C4	
Clinical finding:	r	P	r	P	r	P
SBP_{min}	-0.28	0.1	0.232	0.07	-0.94	0.48
$DBP_{min,}$	**-0.33**	**0.013**	**0.37**	**0.004**	0.076	0.57
$Diur_{min}$	-0.3	0.82	**0.38**	**0.003**	0.182	0.17
$Diur_{max}$	**0.41**	**0.002**	0.15	0.26	-0.128	0.339
Change in weight	**0.42**	**<0.001**	**-0.32**	**0.012**	-0.149	0.255
Treatment time at hospital	**0.46**	**<0.001**	**-0.3**	**0.025**	-0.137	0.296
Laboratory finding:						
Leukocyte $count_{max}$	**0.43**	**<0.001**	**-0.3**	**0.020**	-0.194	0.137
B-Hcr_{min}	-0.24	0.07	**0.52**	**<0.001**	0.1	0.45
B-Hcr_{max}	**0.3**	**0.024**	0.046	0.73	0.01	0.942
Platelet $count_{min}$	**-0.32**	**0.017**	**0.3**	**0.020**	0.004	0.976
S-$Crea_{max}$	**0.27**	**0.03**	-0.183	0.162	-0.134	0.307
IL-6_{max}	0.14	0.34	**-0.62**	**<0.001**	-0.246	0.089
S-Na_{min}	-0.16	0.25	**0.44**	**<0.001**	0.059	0.67

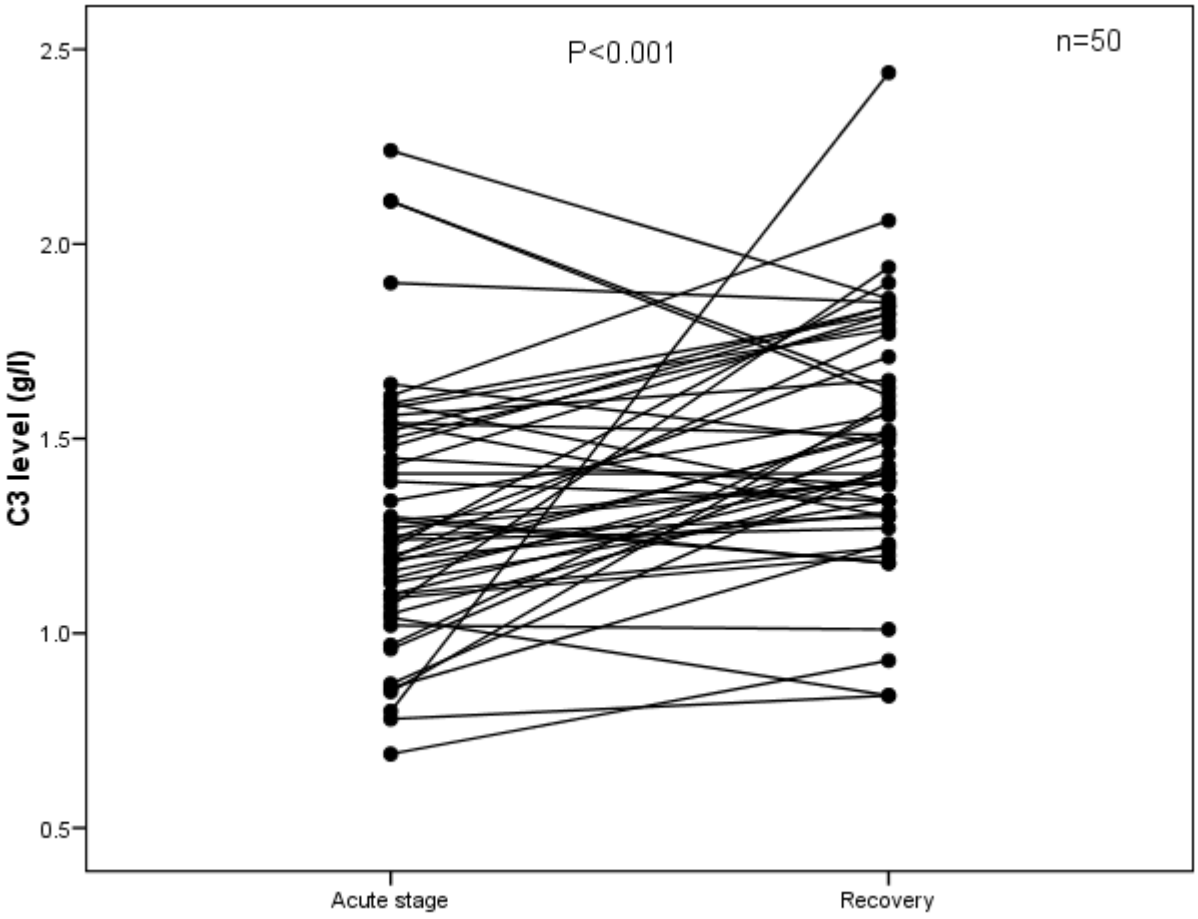
448 **NOTE**. r= correlation coefficient, SBP_{min}= lowest systolic blood pressure, DBP_{min}= lowest diastolic blood
449 pressure, $Diur_{min}$= lowest daily urinary output, $Diur_{max}$= highest daily urinary output, Leukocyte $count_{max}$
450 =highest blood leukocyte count, B-Hcr_{min}=lowest blood hematocrit, B-Hcr_{max}= highest blood hematocrit,
451 Platelet $count_{min}$= lowest blood platelet count, S-$Crea_{max}$= highest serum creatinine concentration,
452 $IL6_{max}$=highest plasma Interleukin-6 level, S-Na_{min}= lowest serum sodium level. Significant correlations are
453 highlighted in bold.

Figures

Figure 1. (A) Paired plasma levels of SC5b-9 of PUUV-HFRS patients during the acute stage upon admission to hospital and at full recovery. The median maximum SC5b-9 concentration in the acute stage was 493 ng/ml (range 103- 1034 ng/ml) and the level at full recovery was 197 ng/ml, (range 100- 522 ng/ml).



462 (B) Paired plasma levels of C3 of PUUV-HFRS patients during the acute stage and at full
463 recovery. The median minimum C3 concentration in the acute stage was 1.26 g/l (range 0.65-
464 2.24 g/l) and the level at full recovery was 1.47 g/l (range 0.84-2.44 g/l).



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(C) Paired plasma levels of C4 of PUUV-HFRS patients during the acute stage and at full recovery. The median minimum C4 concentration in the acute stage was 0.26 g/l (range 0.08-0.52 g/l) and the level at full recovery was 0.27 g/l (range 0.09-1.1 g/l). Wilcoxon's test was used to determine the statistical significance in (A), (B) and (C).

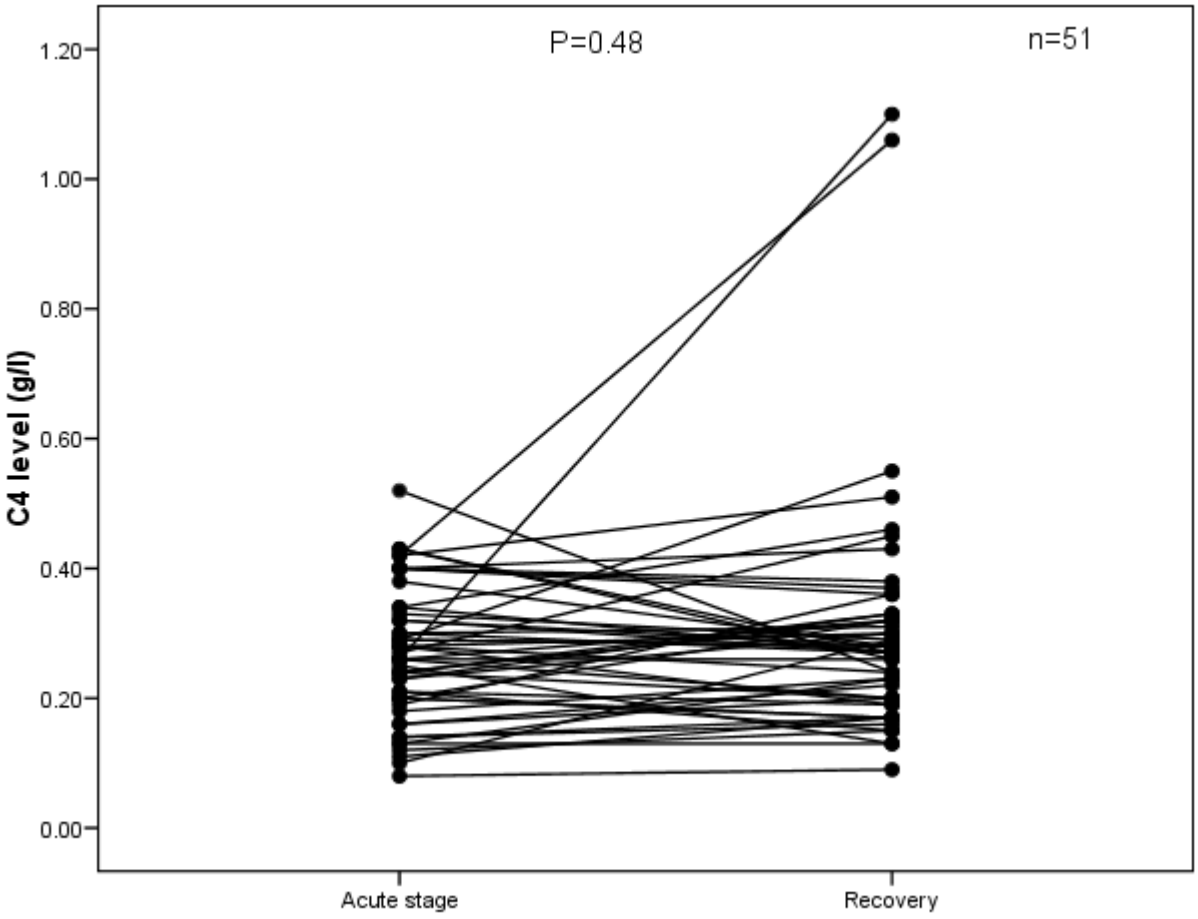
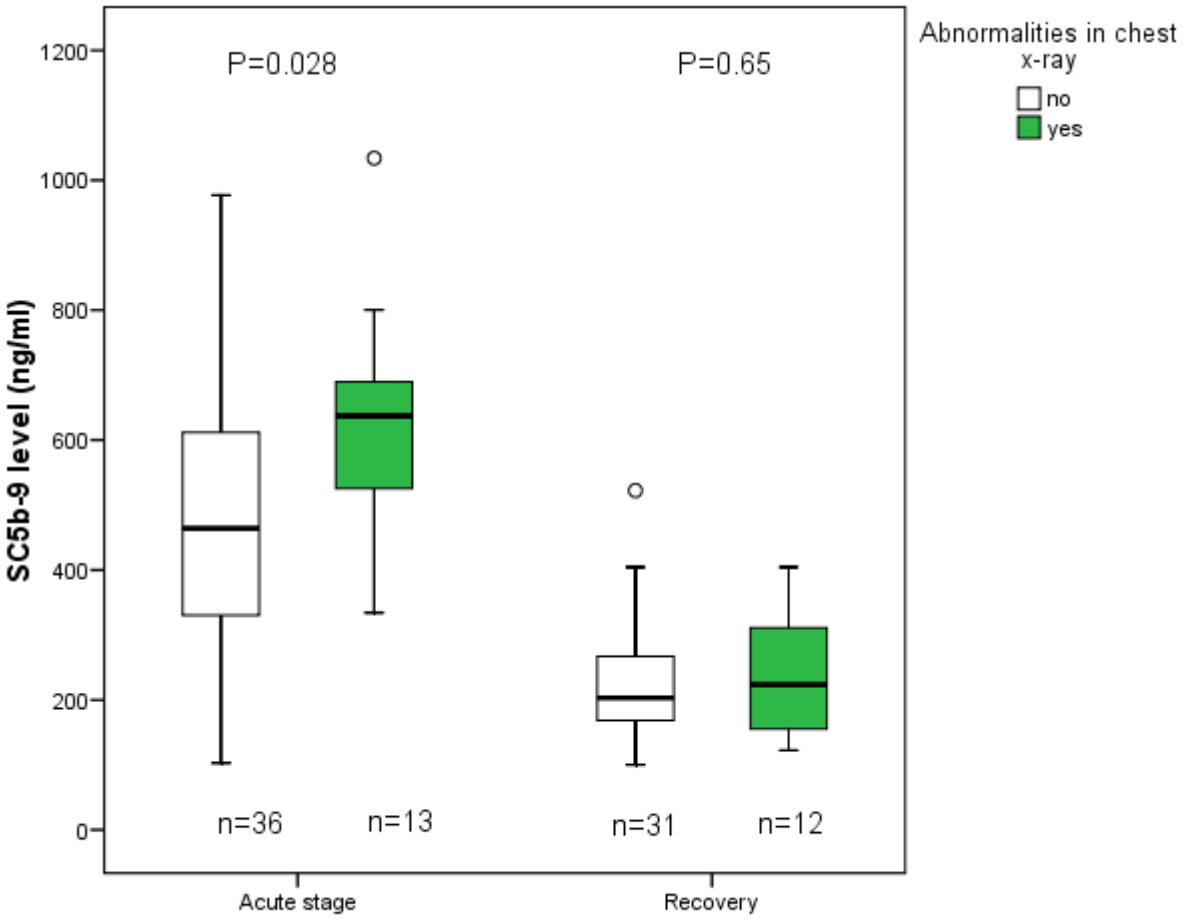
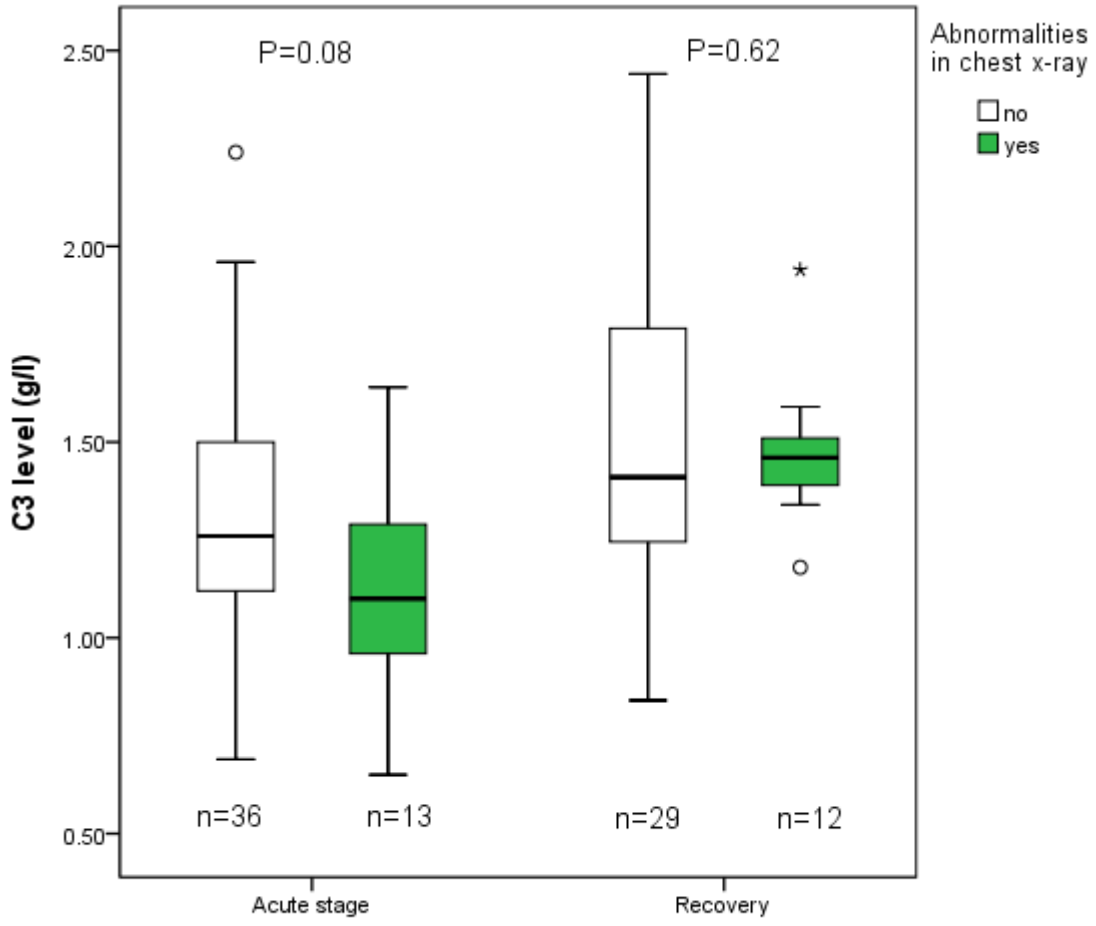


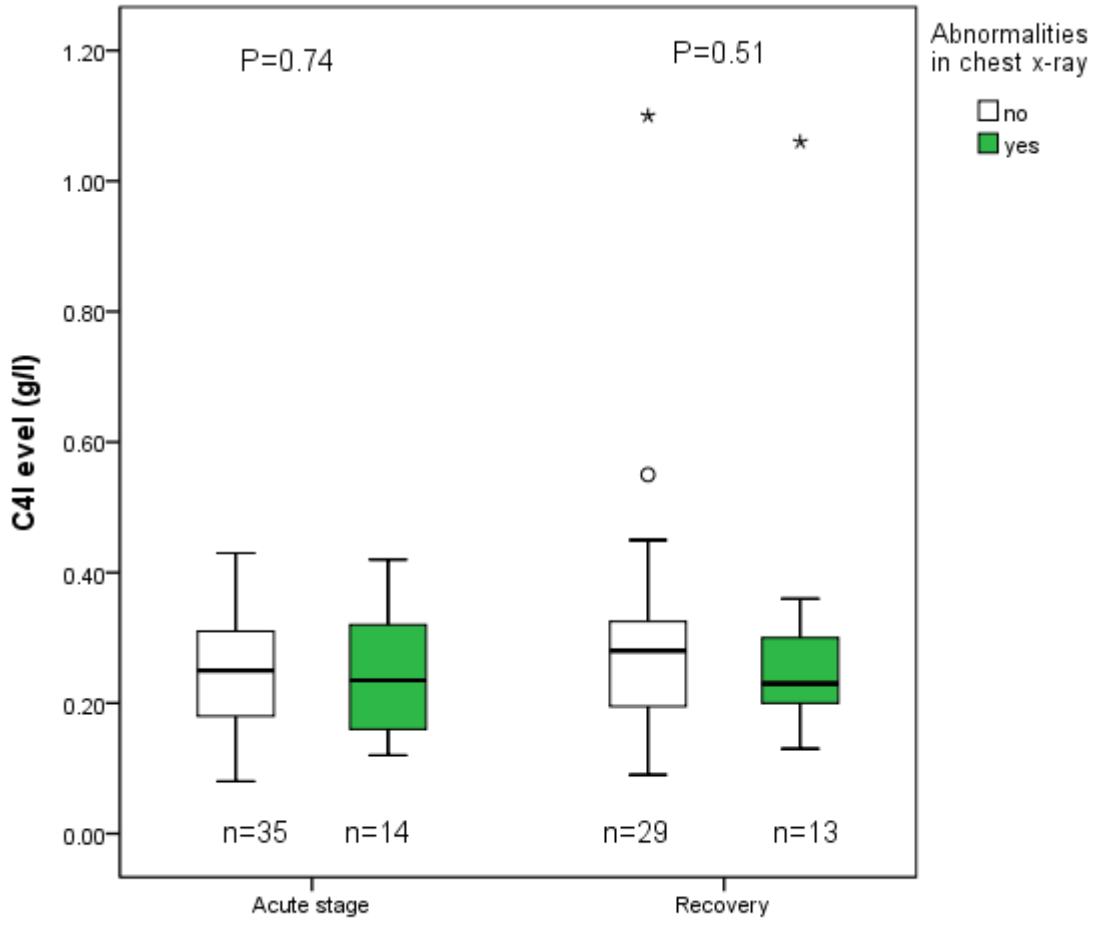
Figure 2. (A) Box plots of plasma SC5b-9 levels of patients with and without chest x-ray abnormalities in the acute stage of PUUV-HFRS and at full recovery. Box plot illustrate the median value (center horizontal line), interquartile range (the lower and upper quartiles), and the highest and lowest values (whiskers) that are not outliers. The outliers are presented as circles. The median maximum SC5b-9 levels in patients with and without chest x-ray abnormalities in the acute stage were 637 ng/ml (range 334-1034 ng/ml) and 464 ng/ml (range 103-977 ng/ml) and at full recovery 222 ng/ml (range 123-365) and 203 ng/ml (100-522 ng/ml), respectively.



(B) Box plots of plasma C3 levels of patients with and without chest x-ray abnormalities in the acute stage of PUUV-HFRS patients and at full recovery. Box plot illustrates the median value (center horizontal line), interquartile range (the lower and upper quartiles) and the highest and lowest values (whiskers) that are not outliers. The outliers are presented as circles (minor outlier) or asterisks (major outlier). The median minimum C3 levels in patients with and without chest x-ray abnormalities in the acute stage were 1.1 g/l (range 0.65-1.64) and 1.26 g/l (range 0.7-2.24) and at full recovery 1.46 g/l (range 1.2-1.94) and 1.41 (range 0.84-2-44), respectively.



(C) Box plots of plasma C4 levels of patients with and without chest x-ray abnormalities in the acute stage of PUUV-HFRS and at full recovery. Box plot illustrate the median value (center horizontal line), interquartile range (the lower and upper quartiles), and the highest and lowest values (whiskers) that are not outliers. The outliers are presented as circles. The median minimum C4 levels in patients with and without chest x-ray abnormalities in the acute stage were 0.24 g/l (range 0.12-0.42 g/l) and 0.25 g/l (range 0.08-0.43 g/l) and at full recovery 0.23g/l (range 0.13-1.06 g/l) and 0.28 g/l (range 0.09-1.10), respectively. Mann Whitney test was used to determine statistical significance in (A), (B) and (C).



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Regular Article

Polymorphisms of PAI-1 and platelet GP Ia may associate with impairment of renal function and thrombocytopenia in Puumala hantavirus infection

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ABSTRACT

Introduction: Puumala virus (PUUV) infection is a viral hemorrhagic fever with renal syndrome (HFRS) characterized by thrombocytopenia and acute impairment of renal function. We aimed to assess whether genetic polymorphisms of platelet antigens together with those of von Willebrand factor (VWF) and plasminogen activator inhibitor (PAI-1) correlate with disease severity.

Patients and methods

172 consecutive hospital-treated patients with serologically confirmed acute PUUV infection were included. Platelet glycoprotein (GP) IIIa T>C (rs5918), GP Ia T>C (rs1126643), GP Ib C>T (rs6065), GP VI T>C (rs1613662), VWF A>G (rs1063856) and PAI-1 A>G (rs2227631) were genotyped. The associations of the rarer alleles with variables reflecting the severity of the disease were analyzed.

Results: PAI-1 G-carriers had higher maximum creatinine level compared with the non-carriers (median 213 μmol/l, range 60–1499 μmol/l vs. median 122 μmol/l, range 51–1156 μmol/l, $p=0.01$). The GG-genotypes had higher creatinine levels than GA- and AA-genotypes (medians 249 μmol/l, 204 μmol/l and 122 μmol/l, respectively, $p=0.03$). Polymorphisms of GP VI and VWF associated with lower creatinine levels during PUUV infection. The minor C-allele of GP Ia associated with lower platelet counts (median 44×10^9/l, range 20–90×10^9/l vs median 64×10^9/l, range 3–238×10^9/l; $p=0.02$).

Conclusions: Polymorphism of PAI-1, a major regulator of fibrinolysis, has an adverse impact on the outcome of kidney function in PUUV-HFRS. Platelet collagen receptor GP Ia polymorphism associates with lower platelet count.

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. [1] In Finland the average annual incidence is 31/100 000 population and the incidence tends to increase. [2] The course of the disease may be divided into febrile, hypotensive, oliguric, diuretic and convalescent phases, but these phases may overlap, and the clinical severity varies. Renal involvement results in the need for transient hemodialysis treatment in 5% of hospital-treated NE patients. [1] Thrombocytopenia is a characteristic finding but bleeding manifestations usually remain mild. [3] The case fatality rate is 0.08%. [2]

The pathogenesis of NE is complex and multifactorial with host genetic properties having an impact on disease severity. Human leukocyte alloantigen (HLA) B8 and DRB1*0301 alleles are associated with severe clinical disease, and genetic polymorphisms of the cytokines tumor necrosis factor alpha, interleukin-1, and interleukin-1 receptor antagonist also influence the outcome of infection. [4,5] The surface density of platelet β_3 integrin, the cellular receptor of hantaviruses, is associated with disease severity in patients with Hantaan virus. [6] However, a human platelet alloantigen (HPA) -1 polymorphism which affects

Abbreviations: PUUV, Puumala virus; HFRS, hemorrhagic fever with renal syndrome; VWF, von Willebrand factor; PAI, plasminogen activator inhibitor; GP, glycoprotein; NE, nephropathia epidemica; HLA, human leukocyte antigen; HPA, human platelet alloantigen; CBC, complete blood count; CRP, C-reactive protein; IL-6, interleukin-6; SNP, single nucleotide polymorphism.

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platelet glycoprotein (GP) IIIa carrying subunit β_3 was not associated with clinical disease severity. Instead, an association of HPA-3 with the severe form of Hantaan virus infection was reported. [7]

In the acute phase of NE, hemorrhagic problems together with thrombocytopenia and altered coagulation are known to occur, but the data published on platelet function and coagulation activity remain limited. We recently reported decreased levels of natural anticoagulants, shortened thrombin time and enhanced fibrinolysis in patients with acute NE. [8] We also found that the levels of three main platelet ligands for $\alpha IIb\beta_3$ (subunit $\beta_3$integrin) were altered, i.e. von Willebrand factor (VWF) and fibrinogen were increased and plasma fibronectin was decreased during acute NE. [9]

Here we studied the genetic polymorphisms of the main antigens involved in platelet activation and aggregation, namely glycoprotein (GP) IIIa (HPA-1), GP Ib (HPA-2), GP Ia (HPA-5) and GP VI, together with VWF and plasminogen activator inhibitor (PAI) -1 in patients with acute NE. Our aim was to establish a possible association between these polymorphisms and the clinical course of NE.

Methods

Patients

The study was carried out at Tampere University Hospital and University of Tampere School of Medicine. All patients came from the Pirkanmaa region and were hospitalized at Tampere University Hospital due to serologically confirmed acute PUUV infection during the period from September 1997 to February 2009. [10] Written informed consent was obtained from all patients, and The Ethics Committee of Tampere University Hospital approved the study protocol.

The study group was comprised of 172 prospectively collected, consecutive patients (118 males), median age 40 years (ranging from 15 to 74 years). Concomitant diseases included arterial hypertension (n = 12), dyslipidemia (n = 7), coronary heart disease (n = 5), bronchial asthma (n = 6), atrial fibrillation (n = 3) and rheumatoid arthritis (n = 3). There were patients with celiac disease, inflammatory bowel disease, valvular heart disease or neurological disease (n = 2 for each).

Clinical and basic laboratory data

The following variables were recorded: the number of days from the onset of illness (i.e. fever) before the first blood test was taken, the length of hospital stay (days), clinical diagnosis of 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 maximum change in weight (kg). The last variable reflects fluid retention during the hospital stay in the oliguric phase of the disease. Complete blood count (CBC), plasma C-reactive protein (CRP) and plasma creatinine were measured according to the clinical indications, and were determined at the Laboratory Centre of the Pirkanmaa Hospital District using standard methods. Plasma interleukin-6 (IL-6) samples were collected up to five consecutive days after hospitalization and determined from 118 patients using a commercially available ELISA kit following the manufacturer's instructions (PeliKine Compact ™ human IL-6 kit, Central Laboratory of The Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). The detection limit for the IL-6 assay was 0.4 pg/ml. The highest or the lowest individual value of the various variables measured during hospitalization was designated as the maximum or the minimum value.

Genotyping

DNA was extracted from whole blood using a commercially available kit (QIAGEN Inc., Hilden, Germany). The gene polymorphisms of GP IIIa (HPA-1) T > C (rs5918) and GP Ib (HPA-2) C > T (rs6065) were genotyped with Assay-By-Design from Applied Biosystems under standard conditions using the ABI Prism 7900HT Sequence Detection System (Taqman, Applied Biosystems, Foster City, CA, USA). The GP Ia (HPA-5) T > C (rs1126643) and the VWF A > G (rs1063856) were genotyped as previously described. [11,12] Genotyping of the GP VI T > C (rs1613662) was performed with minor modifications. [13] For PAI-1 A > G (rs2227631) a commercial kit from Applied Biosystems was used. The distributions of all single nucleotide polymorphisms (SNP) did not deviate from the Hardy–Weinberg equation. Genotyping was successful in 162 of 172 patients (94%) for GP IIIa, in all 172 patients for GP Ia, in 169 patients (98%) for GP Ib, in 171 patients (99%) for GP VI, in 162 patients (94%) for VWF and in 169 patients (98%) for PAI-1.

Statistical analysis

Since all continuous variables were skewed, medians and ranges were calculated to describe the data. Percentages were used for categorical variables. The allele frequencies were counted and the patients were divided in to the groups of carriers and non-carriers of the rarer (minor) allele. Comparisons between the groups are based on Mann–Whitney U or Kruskal-Wallis test for numerical and χ^2 or Fisher's exact test for categorical data. The limit of significance was set at 0.05 (2-tailed). If Bonferroni adjustments for multiple tests were applied, the limit of significance would be set at 0.01. SPSS version 14.0 was used for computation.

Results

Clinical and laboratory findings

All patients suffered from clinically typical PUUV infection. All patients were examined and hospitalized in the acute phase of the illness. The clinical findings and the basic laboratory values are shown in Table 1. Of all patients, 97% had their platelet count minimum below 150 × 10 /l, the lower limit of normal range. There was no correlation between the minimum platelet count and the maximum plasma creatinine level. Three out of 172 patients (2%) were in clinical shock at the time of admission. Seven patients (4%) needed transient hemodialysis treatment. All patients recovered.

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

Clinical or laboratory variable	Median	Range
Days from onset of illness*	4	1-14
Length of hospital stay (days)	6	2-15
Systolic BP min (mmHg)	113	74-170
Diastolic BP min (mmHg)	70	40-100
Daily urinary output min (ml)	1450	50-7000
Change in weight max (kg)**	2.1	0.0-12.0
Hematocrit min	0.36	0.25-0.46
Platelet count min ($x10^9$/l)	62	3-238
Leukocyte count max ($x10^9$/l)	10	3.9-31.2
CRP max (mg/ml)	75	11-269
Interleukin-6 max (pg/ml)	14.5	1.3-107
Creatinine max (μmol/l)	185	51-1499

Abbreviations: min = minimum, max = maximum. References: CRP <10 mg/l, creatinine <105 μmol/l for males and <95 μmol/l for females, platelet count 150–360 and leukocyte count 3.4-8.2 x 10^9/l, hematocrit 0.35-0.50 for males and 0.35-0.46 for females.

* Days from onset of illness equals to the number of days of fever before the first blood test was taken.

** Change in weight during hospital stay reflects the amount of fluid accumulating in the body during the oliguric phase.

Polymorphisms and their associations with the clinical and laboratory findings

Table 2 presents the genotype distributions and allele frequencies of GP IIIa (HPA-1), GP Ib (HPA-2), GP Ia (HPA-5), GP VI, VWF and PAI-1. GP Ib (HPA-2) genotypes did not correlate with any of the variables measured (data not shown).

Renal Function

Renal function impairment in the acute phase of the disease associated with the rare G-allele of PAI-1 gene. The carriers of PAI-1 gene G-allele had 1.7 times higher maximum level of creatinine than the non-carriers (median 213 μmol/l, range 60–1499 μmol/l vs. median 122 μmol/l, range 51–1156; p = 0.01; Fig. 1A). Also, the level of creatinine exceeded 300 μmol/l 1.7 times more often in the G-carriers compared with the non-carriers (38% vs. 22%, p = 0.04). As shown in Table 3, GG-homozygotes had the highest maximum level of creatinine of all PAI-1 carrier genotypes, followed by AG-heterozygotes and AA-homozygotes (median 249 μmol/l, median 204 μmol/l and median 122 μmol/l, respectively, p = 0.03). Six out of seven hemodialysis patients were G-allele carriers, and one of them was a GG-homozygote.

The rare G-allele of VWF A > G was associated with a lower level of maximum creatinine (median 131 μmol/l, range 52–1285 μmol/l vs. median 241 μmol/l, range 51–1499 μmol/l; p = 0.02). Accordingly, the level of creatinine exceeded 300 μmol/l less often in the carriers of the VWF G-allele than in the non-carriers (21% vs. 38%, p = 0.03).

Analyses of genotype combinations revealed that the patients (n = 18, 11% of all patients) carrying the favorable VWF G-allele and not carrying the unfavorable PAI-1 G-allele had lower levels of creatinine compared with the patients not carrying the VWF G-allele and carrying the PAI-1 G-allele (n = 73, 46% of all patients; 99 μmol/l vs. 250 μmol/l, respectively, p = 0.002).

The carriers of the GP VI gene rare C-allele had maximum creatinine levels > 300 μmol/l less often than non-carriers (17% vs. 36%, p = 0.03).

Platelet count

The platelet count nadir was lower in carriers of the GP Ia (HPA-5) gene rare C-allele compared to non-carriers (median 44 × 10 /l, range 20–90 × 10^9/l vs. median 64 × 10 /l, range 3–238 × 10^9/l; p = 0.02). The minor G-allele carriers of PAI-1 gene turned out to have a higher platelet count nadir compared with non-carriers (median 65 × 10 /l, range 9–238 × 10^9/l vs. median 54 × 10 /l, range 3–187 × 10^9/l; p = 0.04; Fig. 1B). Minimum platelet counts by genotypes of GP Ia (HPA-5) and PAI-1 are presented in Table 4.

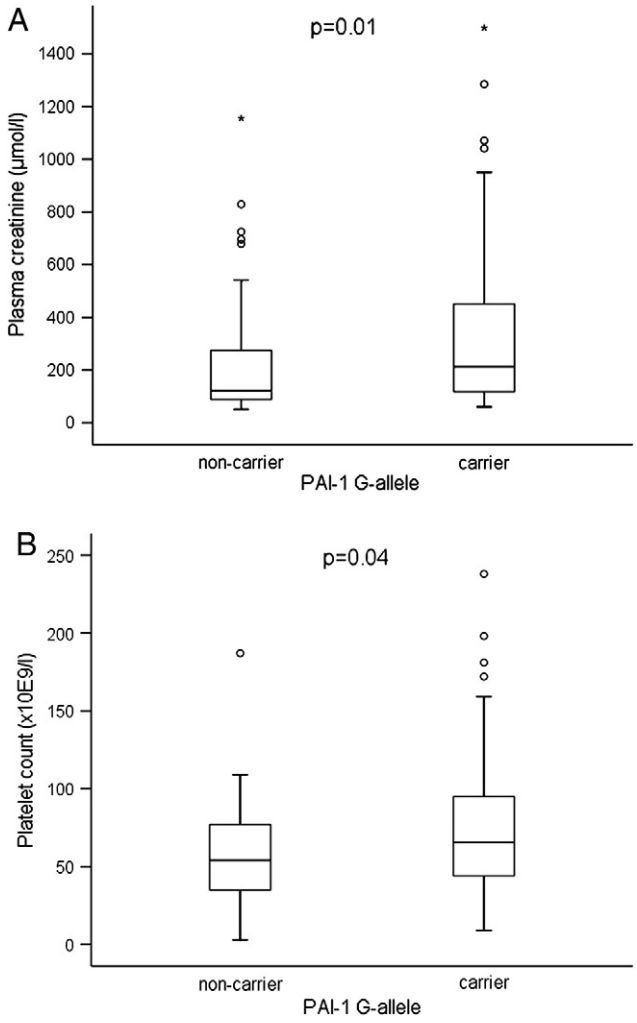

Fig. 1. Box plots of the highest plasma creatinine (A) and the blood platelet count nadir (B) in non-carriers (n = 54) and carriers (n = 115) of the plasminogen activator inhibitor (PAI-1) G-allele (rs 2227631) in 169 patients with PUUV infection. Box plot illustrates the median, the 25% and 75% percentiles, and the lowest and highest values that are not outliers. Outliers are indicated by open circles and extreme values by asterisks.

Inflammation

The maximum level of plasma IL-6 was lower in carriers of the rare GP IIIa (HPA-1) C-allele than in non-carriers (median 13.0 pg/ml, range 1.3-60 pg/ml vs. median 16.5 pg/ml, range 2.6-107 pg/ml; p = 0.05). There were no significant associations with GP IIIa (HPA-1) genotype and CRP or leukocyte count (data not shown).

We found no statistically significant differences in the length of hospital stay, lowest blood pressure, maximum weight change, need

Table 2
The genotype distributions and allele frequencies of platelet GP IIIa, GP Ib, GP Ia, GPVI, VWF and PAI-1 in 172 patients with acute Puumala hantavirus infection.

Polymorphism	Genotype %			Allele %	
	homozygote common	heterozygote	homozygote rare	common	rare
GP IIIa (HPA-1)	66 (TT)	32 (TC)	2 (CC)	82 (T)	18 (C)
GP Ib (HPA-2)	78 (CC)	21 (CT)	1 (TT)	88 (C)	12 (T)
GP Ia (HPA-5)	93 (TT)	7 (TC)	0 (CC)	96 (T)	4 (C)
GP VI T > C (rs1613662)	79 (TT)	19 (TC)	2 (CC)	89 (T)	11 (C)
VWF A > G (rs1063856)	68 (AA)	25 (AG)	7 (GG)	81 (A)	19 (G)
PAI-1 A > G (rs227631)	32 (AA)	53 (AG)	15 (GG)	59 (A)	41 (G)

Abbreviations: GP = glycoprotein, HPA = human platelet alloantigen, VWF = von Willebrand factor, PAI-1 = plasminogen activator inhibitor.

Table 3
Maximum creatinine levels in patients with acute Puumala hantavirus infection presented by genotypes of GP VI T > C (rs1613662), VWF A > G (rs1063856) and PAI-1 A > G (rs227631) polymorphisms.

Polymorphism	Maximum creatinine μmol/l, median (range)			
	Homotsygote common	Heterotsygote	Homotsygote rare	p-value
GPVI T > C (rs1613662)	199 (52–1499)	191 (51–679)	137 (110–196)	0.381
VWF A > G (rs1063856)	241 (51–1499)	125 (52–1285)	137 (60–384)	0.072
PAI A > G (rs227631)	122 (51–1156)	204 (60–1499)	249 (65–906)	0.033

Table 4
Minimum platelet counts in patients with acute Puumala hantavirus infection presented by genotypes of GP Ia T>C (HPA-5) and PAI-1 A>G (rs227631) polymorphisms.

Polymorphism	Minimum platelet count x 10^9/l, median (range)			
	Homotsygote common	Heterotsygote	Homotsygote rare	p-value
GP Ia T>C (HPA-5)	64 (3–238)	44 (20–90)	-	0.023
PAI-1 A>G (rs227631)	54 (3–187)	65 (9–181)	71 (14–238)	0.108

for transient hemodialysis treatment or occurrence of shock compatible with polymorphisms studied here (data not shown).

Discussion

In our study, carriers of the PAI-1 gene minor G-allele suffered from more severe renal impairment than non-carriers. Both the maximum level of creatinine and the proportion of patients having their creatinine value > 300 μmol/l were higher in the carriers of G-allele compared with non-carriers. GG-homozygotes had the highest creatinine levels, and 6/7 patients who needed transient hemodialysis treatment were G-allele carriers. The platelet count nadir of the PAI-1 G-allele carriers was higher than that of non-carriers, and was concordant with our observation that the severity of renal impairment does not associate with the depth of thrombocytopenia in acute PUUV infection.

PAI-1 is secreted by a variety of cells including platelets and endothelial cells in response to inflammatory stimuli, and PAI-1 is the primary physiological regulator of tissue- and urokinase-type plasminogen activators and fibrinolysis. [14] PAI-1 polypeptide is encoded by the SERPINE1 (SERine Proteinase INhibitor, clade E, member 1) gene on chromosome 7q21.3-q2, and association of the 4 G/5 G polymorphism (rs1799889) with increased PAI-1 levels of 4 G-allele carriers has been suggested by several studies. Elevated levels of PAI-1 have been related to poor outcome in several infections such as pneumonia, sepsis, meningococcal disease, and dengue virus infection. [15-21] The 4 G-allele also seems to associate with atherosclerotic diseases of coronary arteries and reduced kidney graft survival. [14,22] We studied the SNP rs2227631 of the PAI-1 gene which is very close to 4 G/5 G polymorphism (rs1799889) and has a relatively high correlation with 4 G/5 G. [14,23] We found that the carriers of the rare G-allele suffered from more severe renal failure during the acute phase of NE compared with non-carriers. Unfortunately, we could not measure the plasma levels of PAI-1, and to our knowledge, there are no data on the plasma level and role of PAI-1 in hantavirus infections. Thus the plausible increase of PAI-1 level in response to infection and acute phase reaction and its modification by polymorphisms of PAI-1 gene in hantavirus infection remain the subjects of further studies.

The hemostatically active ligand VWF plays a critical role in platelet aggregation and adhesion to injured endothelium during primary hemostasis and pathologic thrombus formation. The VWF gene is highly polymorphic, and genetic factors are responsible for up to 66% of the variation in plasma VWF levels. [24] We recently observed high plasma VWF concentrations during acute PUUV infection. [9] Here we studied the A>G polymorphism of the WVF gene (rs 1063856) in exon 18 of chromosome 12. This polymorphism has been associated with high VWF levels, and is close to SNP rs10638857 which is known to associate with VWF levels in healthy subjects. [24-26] We found the minor G-allele (rs 1063856) of the VWF gene to have some protective role in kidney function, but we were unable to conclude whether this favorable effect depends on modulation of plasma VWF levels.

Binding of collagen to GP VI leads to platelet activation and procoagulant activity contributing to the formation of the hemostatic plug. Low frequency allele homozygotes of GP VI, such as the CC genotype of T>C rs 1613662, are associated with reduced activation and platelet responses to collagen when compared with high frequency allele homozygotes. [27] In this study, the rare C-allele of GP VI offered some protection of renal function, but did not correlate with the platelet count or other variables of disease severity. A lower platelet count nadir was associated with the low frequency C-allele of GP Ia (HPA-5), the other major platelet receptor for collagen. [28] However, the associations between genetic polymorphisms and platelet function could not be studied here.

GP IIbIIIa complex mediates platelet aggregation by binding the adhesive proteins VWF, fibrinogen, and thrombus stabilizing fibronectin. [29] GP IIbIIIa carries the HPA-1 polymorphism site and contains subunit β_3 integrin (IIIa), the receptor for hantaviruses.[30,31] We found that carriers of the low frequency C-allele of GP IIIa (HPA-1) had slightly lower levels of plasma IL-6, a variable known to positively correlate with the severity of PUUV infection.[32] Our findings are similar to the only previous study on hantavirus infection and GP IIIa polymorphism which found that the HPA-1 polymorphism does not associate with disease severity.[7]

Due to the design of the study, we were not able to obtain reliable information about bleeding symptoms, plasma levels of PAI-1 and VWF, or platelet functions. However, for the first time, genetic polymorphisms related to platelet activation, blood coagulation and fibrinolysis were shown to have an impact on kidney function and platelet count in HFRS. Future studies could clarify the role of each of these genetic polymorphisms, their synergistic effects, and their functional relevances in PUUV-induced HFRS.

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Conflict of interest statement

The authors have no conflict of interest.

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