TUULA OUTINEN

Biomarkers for Predicting the Outcome of Puumala Hantavirus Infection

ACADEMIC DISSERTATION
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To my loved ones

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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 Outinen TK, Mäkelä S, Ala-Houhala I, Huhtala H, Hurme M, Paakkala A, Pörsti I, Syrjänen J, Mustonen J: The severity of Puumala hantavirus induced nephropathia epidemica can be better evaluated using plasma interleukin-6 than C-reactive protein determinations. BMC Infect Dis 2010; 10:132.

II Outinen TK, Mäkelä S, Huhtala H, Hurme M, Meri S, Pörsti I, Sane J, Vaheri A, Syrjänen J, Mustonen J: High pentraxin-3 plasma levels associate with thrombocytopenia in acute Puumala hantavirus-induced nephropathia epidemica. Eur J Clin Microbiol Infect Dis 2012; 31:957-964.

III Outinen TK, Mäkelä S, Ala-Houhala I, Huhtala H, Hurme M, Libraty D, Oja SS, Pörsti I, Syrjänen J, Vaheri A, Mustonen J: High activity of indoleamine 2,3-dioxygenase is associated with renal insufficiency in Puumala hantavirus induced nephropathia epidemica. J Med Virol 2011; 82:731-737.

IV Outinen TK, Kuparinen T, Jylhävä J, Leppänen S, Mustonen J, Mäkelä S, Syrjänen J, Vaheri A, Hurme M: Plasma cell-free DNA levels are elevated in acute Puumala hantavirus infection. PLoS One 2012; 7(2):e31455.

In addition, this thesis contains unpublished data. The original publications are reproduced in this thesis with the permission of the copyright holders.

ABBREVIATIONS

ANDV	Andes virus
ARF	Acute renal failure
AUC	Area under curve
BMI	body mass index
bp	base pair
cf-DNA	cell-free deoksiribonucleic acid
CNS	central nervous system
CRP	C-reactive protein
CT	computed tomography
DIC	disseminated intravascular coagulopathy
CMV	cytomegalovirus
DNA	deoksiribonucleic acid
DOBV	Dobrava virus
EBV	Epstein-Barr virus
ECG	electrocardiogram
ECHO	echocardiography
HBV	hepatitis B virus
HCV	hepatitis C virus
HCPS	hantavirus cardiopulmonary syndrome
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTNV	Hantaan virus
IDO	indoleamine 2,3-dioxygenase
IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MRI	magnetic resonance imaging
MRR	magnetic resonance renography
N	nucleocapsid
NE	nephropathia epidemica
NK	natural killer
PRR	pattern recognition receptor
PTX3	pentraxin-3
PUUV	Puumala virus
RNA	ribonucleic acid
ROC	receiver operating characteristic
RSV	respiratory syncytial virus
SAAV	Saaremaa virus
SARS	severe acute respiratory syndrome
SEOV	Seoul virus

SNV	Sin Nombre virus
sIL-2R	soluble interleukin-2 receptor
SLE	systemic lupus erythematosus
TEC	tubular epithelial cell
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T lymphocyte
US	ultrasound
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor-2

ABSTRACT

Puumala hantavirus (PUUV) causes a mild type of hemorrhagic fever with renal syndrome called nephropathia epidemica (NE). After an incubation period of 1-8 weeks, NE presents with sudden high fever, headache, nausea, abdominal pain, backache, visual disturbances, and impaired renal function. The severity of NE varies from asymptomatic to rare fatal cases, and its pathogenesis is not completely understood. An important feature in hantaviral infections is capillary leakage due to increased capillary permeability. The mechanisms behind this phenomenon are unclear, although immunological responses have been suggested to be important.

In the present study, the association of immunological factors, i.e. interleukin (IL)-6, C-reactive protein (CRP), pentraxin-3 (PTX3), indoleamine 2,3-dioxygenase (IDO), and cell-free DNA (cf-DNA), with the severity of NE was analyzed. Furthermore, their possible role in the pathogenesis was assessed.

Pentraxins are a family of acute-phase proteins. CRP is a short pentraxin mainly produced in the liver in response to inflammatory signals. IL-6, in turn, is a multifunctional cytokine involved in immune responses and inflammation. Increased cytokine levels have previously been found in the plasma, urine, and tissues of patients with hantavirus infection. In Study I, plasma IL-6 and CRP as well as their association with disease severity reflecting variables were studied in 118 hospital-treated patients with acute NE. High plasma IL-6 levels were found to associate with clinically severe acute NE. High IL-6 levels were also found as an independent risk factor for impaired renal function. High plasma CRP, in turn, did not have an association with a more severe course of the disease. On the contrary, high CRP levels turned out to be a possible protective factor for renal function.

PTX3 is a long pentraxin produced at the site of inflammation. In Study II, 61 hospitalized PUUV-infected patients were studied to assess the associations of plasma PTX3 with variables reflecting the severity of acute NE. PTX3 levels were shown to be elevated during the acute phase of the disease. High PTX3 associated with a more severe course of NE and, most of all, with significant thrombocytopenia. It also associated with the activation of the complement system. Thus, PTX3 could possibly be involved in the pathogenesis of thrombocytopenia in NE through the activated complement cascade.

IDO is the rate-limiting enzyme in tryptophan catabolism to kynurenine leading to depletion of tryptophan as well as T cell suppression. In Study III, 102 hospitalized patients were studied to establish the association of serum IDO enzyme with the variables reflecting the severity of acute NE. Serum tryptophan and kynurenine levels were determined by reverse-phase high-performance liquid chromatography, and tryptophan/kynurenine ratio reflecting IDO activity was calculated. IDO levels were found to be elevated during acute NE. High IDO was revealed to associate with clinically severe NE and it presented as an independent risk factor for significant renal insufficiency. Furthermore, serum IDO levels were shown to peak before serum creatinine levels. It is conceivable that IDO is involved in the pathogenesis of renal insufficiency in PUUV infection. The possible

mechanisms are promotion of tubular epithelial cell apoptosis or immunosuppression through T cell suppression.

Elevated levels of cf-DNA have been previously reported in different clinical disorders. The current view is that, in these conditions, cf-DNA originates from apoptotic or necrotic cells and therefore reflects the amount of cellular damage. In Study IV, total cf-DNA was studied in the plasma of 61 patients and urine of 20 patients with acute NE. Also, a qualitative high-sensitivity lab-on-a-chip DNA assay was carried out in 20 patients to elucidate the appearance of cf-DNA in plasma and urine. The plasma levels of cf-DNA were found to be elevated during acute PUUV infection and correlate with the apoptotic band (150-200 base pairs) intensity. The total plasma cf-DNA concentration also correlated with leukocytosis, thrombocytopenia, and the length of hospitalization. The urinary excretion of cf-DNA, in turn, was not elevated during the acute infection and it did not correlate with any of the disease severity reflecting variables.

In conclusion, high plasma IL-6, PTX3, cf-DNA, and serum IDO levels reflect the clinical severity of NE, while high CRP concentration seems to protect against renal failure and does not predict a severe course of NE. Neither does urinary excretion of cf-DNA reflect the degree of inflammation in the kidney. Furthermore, PTX3 might be involved in the pathogenesis of thrombocytopenia and IDO, in turn, could act in the pathogenesis of renal insufficiency.

TIIVISTELMÄ

Hantaviruksiin kuuluva Puumala-virus aiheuttaa lievän munuaisoireisen verenvuotokuumeen, jota kutsutaan myyräkuumeeksi. Tauti alkaa äkillisesti 1-8 viikon itämisajan jälkeen korkealla kuumeella ja päänsäryllä, joita seuraa pahoinvointi, vatsa- ja selkäkivut, näköhäiriöt sekä munuaisten vajaatoiminta. Puumala-virusinfektion vaikeusaste vaihtelee oireettomasta harvinaisiin kuolemaan johtaviin tapauksiin. Myyräkuumeen taudin kehittymistä ei täysin tunneta. Hantavirusinfektioissa tärkeä piirre on kapillaarien läpäisevyyden lisääntymisestä johtuva kapillaarivuoto. Tämän ilmiön taustalla olevat mekanismit ovat epäselviä, mutta immunologisilla reaktioilla on arveltu olevan tärkeä osuus.

Tässä väitöskirjatyössä tutkittiin immunologisten tekijöiden, interleukiini (IL)-6:n, C-reaktiivisen proteiinin (CRP), pentraksiini-3:n (PTX3), indoleamiini 2,3-dioksygenaasin (IDO) ja soluvapaan DNA:n (cf-DNA), yhteyttä myyräkuumeen vaikeusasteeseen. Myös näiden tekijöiden mahdollista osuutta taudin kehittymisessä arvioitiin.

Pentraksiinit ovat ryhmä akuutin faasin proteiineja. CRP on lyhyt pentraksiini, jota tuotetaan pääasiassa maksassa vasteena tulehduksellisille signaaleille. IL-6 puolestaan on sytokiini, jolla on useita tehtäviä ja joka on osallisena immuunivasteen säätelyssä ja tulehdusreaktioissa. Lisääntyneitä sytokiinipitoisuuksia on aiemmin todettu hantavirusinfektiopotilaiden plasmassa, virtsassa ja kudoksissa. Osatyössä I tutkittiin plasman IL-6- ja CRP-pitoisuuksia sekä niiden yhteyttä taudin vaikeusastetta kuvastaviin muuttujiin 118 sairaalahoidetulla myyräkuumepotilaalla. Korkean IL-6-pitoisuuden todettiin liittyvän vaikeaan akuuttiin myyräkuumeeseen. Se osoittautui myös itsenäiseksi riskitekijäksi munuaisten vajaatoiminnalle. Korkea plasman CRP puolestaan ei liittynyt vaikeampaan tautiin. Päinvastoin, korkea CRP osoittautui mahdolliseksi munuaisten toimintaa suojaavaksi tekijäksi.

PTX3 on pitkä pentraksiini, jota tuotetaan tulehduspaikalla. Osatyössä II tutkittiin 61 sairaalahoidettua myyräkuumepotilasta plasman PTX3-pitoisuuden ja taudin vaikeusastetta kuvastavien muuttujien yhteyden selvittämiseksi. PTX3-pitoisuuden todettiin olevan koholla akuutissa myyräkuumeessa. Korkea PTX3-pitoisuus oli yhteydessä vaikeaan myyräkuumeeseen ja erityisesti matalaan verihiutaletasoon. Korkea PTX3 oli yhteydessä myös komplementtijärjestelmän aktivaatioon. Näin ollen PTX3 voisi olla osallisena matalan trombosyyttitason kehittymisessä myyräkuumeessa aktivoituneen komplementtijärjestelmän kautta.

IDO on katabolianopeutta rajoittava entsyymi tryptofaanin pilkkoutumisessa kynureniiniksi, mikä johtaa tryptofaanin puutteeseen ja T-solujen estoon. Osatyössä III tutkittiin seerumin IDO-entsyymin ja myyräkuumeen vaikeusastetta kuvastavien muuttujien yhteyttä 102 sairaalahoidetulla potilaalla. Seerumin tryptofaanin ja kynureniinin pitoisuudet määritettiin ja laskettiin IDO-aktiivisuutta heijastava tryptofaani/kynureniini-suhde. IDO-pitoisuuden todettiin olevan koholla akuutissa myyräkuumeessa. Korkea IDO-pitoisuus oli yhteydessä vaikeaan myyräkuumeeseen ja osoittautui merkittävän munuaisten vajaatoiminnan itsenäiseksi riskitekijäksi.

Lisäksi seerumin IDO-pitoisuus oli korkeimmillaan ennen kreatiniinipitoisuuden huippua. IDO saattaa olla mukana munuaisten vajaatoiminnan kehittymisessä myyräkuumepotilailla. Mahdollisia mekanismeja ovat tubulaaristen epiteelisolujen apoptoosi tai T-solujen eston aiheuttama immuunilama.

Kohonneita soluvapaan DNA:n pitoisuuksia on aiemmin raportoitu erilaisissa sairauksissa. Nykykäsityksen mukaan soluvapaa DNA on näissä tiloissa peräisin apoptoottisista tai nekroottisista soluista ja siten kuvastaa solutuhon määrää. Osatyössä IV plasman soluvapaan DNA:n pitoisuus määritettiin 61 myyräkuumepotilaalta ja virtsan soluvapaan DNA:n eritys 20 potilaalta. Lisäksi tehtiin kvalitatiivinen DNA-määritys 20 potilaalle sekä plasmasta että virtsasta soluvapaan DNA:n ulkomuodon selvittämiseksi. Plasman soluvapaan DNA:n pitoisuudet todettiin koholla oleviksi akuutissa myyräkuumeessa ja ne korreloivat apoptoottisen juosteen (150-200 emäsparia) voimakkuuden kanssa. Plasman soluvapaan DNA:n kokonaismäärä korreloi myös positiivisesti valkosolutason ja sairaalahoidon keston sekä negatiivisesti verihiutaletason kanssa. Virtsan soluvapaan DNA:n eritys puolestaan ei ollut akuutissa myyräkuumeessa koholla, eikä se korreloinut minkään taudin vaikeusastetta kuvastavan muuttujan kanssa.

Yhteenvetona todetaan, että korkea IL-6, PTX3, IDO ja plasman soluvapaa DNA liittyvät vaikeaan myyräkuumeeseen. Korkea CRP puolestaan näyttäisi suojaavan munuaistoimintaa eikä kuvasta vaikeaa tautia. Virtsan soluvapaan DNA:n eritys ei kuvasta tulehduksen määrää munuaisissa. PTX3 voi olla mukana matalan verihiutaletason kehittymisessä ja IDO puolestaan munuaisten vajaatoiminnan kehittymisessä myyräkuumeessa.

1. INTRODUCTION

Puumala hantavirus (PUUV) causes a mild hemorrhagic fever with renal syndrome (HFRS), called nephropathia epidemica (NE) (Vapalahti et al. 2003). The natural carrier rodent of PUUV is the bank vole (*Myodes glareolus*) (Brummer-Korvenkontio et al. 1980). Other hantaviruses causing HFRS include Hantaan (HTNV), Dobrava (DOBV), Saaremaa (SAAV), Amur, and Seoul (SEOV) viruses (Vapalahti et al. 2003, Heyman and Vaheri 2008). In the Americas, Sin Nombre (SNV), Andes (ANDV), Black Creek Canal, and several other viruses cause hantavirus cardiopulmonary syndrome (HCPS) (Kanerva et al. 1998a). NE occurs in Finland, elsewhere in Scandinavia, in Western Russia, the Balkans, and many parts of Central-Western Europe (Vapalahti et al. 2003). In Finland, 1,000-3,000 serological PUUV infection diagnoses are made annually (THL 2012).

The clinical picture of NE varies from a subclinical disease to rare fatal cases (Makary et al. 2010). Usual symptoms include sudden high fever, headache, abdominal pain, nausea, backache, and visual disturbances, while serious hemorrhagic manifestations are uncommon (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010). Renal involvement causes proteinuria, hematuria, and oliguria, which is followed by polyuria (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010). Minority of patients need transient hemodialysis treatment during the oliguric phase. The characteristic histopathologic renal finding is acute tubulointerstitial nephritis and common laboratory findings include leukocytosis, thrombocytopenia, anemia, and elevation of plasma C-reactive protein (CRP) and creatinine levels (Settergren et al. 1989, Mustonen et al. 1994a, Mustonen et al. 1994b). The pathogenesis of NE is not completely understood. An important feature in hantaviral infections is universally increased capillary permeability, but the mechanisms behind this phenomenon are unclear (Cosgriff 1991). It has been suggested that immunological factors are essential in the pathogenesis of NE (Cosgriff 1991, Kanerva et al. 1998a).

Pentraxins are a family of acute-phase proteins, which are characterized by a cyclic multimeric structure (Bottazzi et al. 2009). CRP is the prototype short pentraxin mainly produced in the liver in response to inflammatory signals (Mantovani et al. 2008, Bottazzi et al. 2009). Interleukin (IL)-6 is the main inducer of CRP production (Ganter et al. 1989). The major functions of CRP are complement activation, enhancement of phagocytosis, and induction of cytokine synthesis (Volanakis 2001, Ablij and Meinders 2002). CRP is widely used in clinical practice in the context of assessing the severity of various infectious diseases. Studies concerning the ability of CRP to predict the severity of the disease in viral infections have produced controversial results.

Pentraxin 3 (PTX3) is the prototype protein of the long pentraxin group members. It is produced by a variety of peripheral tissues and cells, mainly mononuclear phagocytes and dendritic cells, in response to pro-inflammatory signals, such as IL-1β, tumor necrosis factor (TNF)-α and Toll-like receptor (TLR) activation (Mantovani et al. 2006, Mantovani et al. 2008, Bottazzi et al. 2009). PTX3 can interact with a number of selected bacteria, fungi and viruses, promoting phagocytosis and clearance of the microbe (Deban et al. 2009). It has the capacity to bind complement component C1q and to participate in the activation of the classical complement pathway (Bottazzi et al. 1997). PTX3 also interacts with factor H, an alternative pathway regulator (Deban et al. 2008). Furthermore, it plays a role in tuning inflammation, in matrix deposition and female fertility (Mantovani et al. 2006, Deban et al. 2009). Previously, in the context of viral infections, PTX3 concentrations have been detected to be higher in patients suffering from dengue shock syndrome than in patients with dengue fever or dengue hemorrhagic fever (Mairuhu et al. 2005).

IL-6 is a multifunctional cytokine involved in immune responses and inflammation. Increased cytokine levels have previously been found in the plasma, urine, and tissues of patients with hantaviral infection (Linderholm et al. 1996, Temonen et al. 1996, Mäkelä et al. 2004). In addition, high IL-6 level has been found to be associated with the severity of NE (Linderholm et al. 1996, Takala et al. 2000, Mäkelä et al. 2004, Sadeghi et al. 2011). In other viral infections, the results concerning IL-6 in the prediction of disease severity have been controversial.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme catalyzing the first and rate-limiting step in the pathway of tryptophan catabolism to kynurenine and its

derivatives (Mellor and Munn 2004, Mellor 2005). IDO is expressed widely in various immune cells, including macrophages and dendritic cells (Mellor and Munn 2004, Mellor 2005). It is also expressed in other types of cells, such as tumor cells, fibroblasts, and renal tubular epithelial cells (TEC) (Mellor and Munn 2004, Mellor 2005, Mohib et al. 2007). Interferon (IFN)-γ is the strongest known inducer of IDO (Mellor and Munn 2004). Increased IDO activity results in the depletion of tryptophan leading to inhibition of T cell responses and proliferation, and thus to immunosuppression and immunotolerance (Hwu et al. 2000, Mellor et al. 2002, Mellor and Munn 2004, Mellor 2005). By reducing tryptophan, IDO activity also inhibits the multiplication of various bacteria and intracellular parasites, and the replication of viruses (Mellor and Munn 2004). Previously, increased IDO activity has been detected in some viral infections, such as dengue virus infection and chronic hepatitis C virus (HCV) infection (Larrea et al. 2007, Becerra et al. 2009). Furthermore, in the case of human immunodeficiency virus (HIV) infection, enhanced tryptophan degradation by IDO was associated with disease progression and complications (Schroecksnadel et al. 2007).

Circulating cell-free DNA (cf-DNA) has recently been studied in various acute and chronic disorders. Elevated levels of cf-DNA have been reported in different conditions, such as in cancer, autoimmune diseases, stroke, myocardial infarction, trauma and sepsis (Lo et al. 2000, Jahr et al. 2001, Rainer et al. 2003, Antonatos et al. 2006, Zhong et al. 2007b, Saukkonen et al. 2008, Mosca et al. 2009, Huttunen et al. 2011b). It has also been suggested that cf-DNA could be used as a predictor of outcome in these conditions (Butt and Swaminathan 2008). Although the concentrations are low, detectable levels of cf-DNA are present also in the plasma of healthy individuals (Zhong et al. 2007a). The current view is that, in different diseases, cf-DNA originates from apoptotic or necrotic cells and therefore reflects the amount of cellular damage (Jahr et al. 2001). Studies on plasma cf-DNA in viral infections are sparse and urine levels of cf-DNA have not previously been studied in viral infections.

In the present study, CRP, PTX3, IL-6, IDO, and cf-DNA were studied in acute PUUV infection. The association of these immunological variables with the severity of the disease was examined as well as their possible role in the pathogenesis of NE.

2. REVIEW OF THE LITTERATURE

2.1 Puumala virus and other hantaviruses

2.1.1 Virology

PUUV was found in the lungs of bank voles (*Myodes glareolus*) collected in the Puumala region, in Finland, in 1977 (Brummer-Korvenkontio et al. 1980). PUUV belongs to the *Hantavirus* genus and the *Bunyaviridae* family (Schmaljohn and Dalrymple 1983, Schmaljohn et al. 1985). Hantaviruses are enveloped RNA viruses possessing a three-segmented negative stranded RNA genome (Schmaljohn and Dalrymple 1983, Schmaljohn et al. 1985). The large (L) segment encodes the viral RNA-dependent RNA-polymerase, which is thought to be responsible for the transcription and replication of the viral genome (Plyusnin 2002). The medium (M) segment encodes the surface envelope glycoproteins Gn and Gc, which are believed to recognize hantavirus receptors on target cells (Plyusnin 2002). Finally, the small (S) segment encodes the nucleocapsid (N) protein, which encapsidates the genome RNA into three viral chromosomes (Plyusnin 2002).

Hantaviruses are maintained in persistently infected rodent hosts and each hantavirus associates predominantly with one specific rodent species (Kanerva et al. 1998a, Plyusnin 2002). The rodent carriers are asymptomatic and excrete the virus in their urine, saliva and feces, thus offering a route of transmission to humans (Lee et al. 1981, Hardestam et al. 2008). The carrier rodent of PUUV, the bank vole, is found throughout Europe, with the exception of the Mediterranean region and the northern parts of Finland, Sweden and Norway (Vapalahti et al. 2003). Hantaviruses cause two clinical syndromes in humans, HFRS and HCPS (Kanerva et al. 1998a, Jonsson et al. 2010). The HFRS causing viruses are distributed in Asia and Europe, whereas the HCPS causing viruses are prevalent in the Americas (Kanerva et al. 1998a, Jonsson et al. 2010). Furthermore, several hantaviruses have not been associated with any human disease. Table 1 shows the hantaviruses associated with

human infections, their rodent hosts, hantaviral clinical syndromes, and geographic distribution.

Table 1. Human pathogenetic hantaviruses, clinical disease, rodent hosts and geographic distribution.

Virus	**Rodent host**	**Distribution**
HFRS causing viruses		
Hantaan	*Apodemus agrarius*	China, Russia, Korea
Dobrava	*Apodemus flavicollis*	Balkans
Seoul	*Rattus norvegicus*	Worldwide
Saaremaa	*Apodemus agrarius*	Europe
Amur	*Apodemus peninsulae*	Russian Far East
Puumala	*Myodes glareolus*	Europe
HCPS causing viruses		
Sin Nombre	*Peromyscus maniculatus*	North America
New York	*Peromyscus leucopus*	North America
Monongahela	*Peromyscus maniculatus numiterrae*	North America
Bayou	*Oryzomys palustris*	North America
Black Creek Canal	*Sigmodon hispidus*	North America
Laguna Negra	*Calomys laucha*	Paraguay, Bolivia, Argentina
Andes	*Oligoryzomys longicaudatus*	Argentina, Chile
Orán	*Oligoryzomys longicaudatus*	Argentina
Choclo	*Oligoryzomys fulvescens*	Panama
Lechiguanas	*Oligoryzomys flavescens*	Argentina
Araraquara	*Bolomys lasiurus*	Brazil
Juquitiba	*Oligoryzomys nigripes*	Brazil
Bermejo	*Oligoryzomys chocoensis*	Argentina
Maciel	*Bolomys obscurus*	Argentina
Muleshoe	*Sigmodon hispidus*	North America
Castelo Dos Sonhos	Unknown	Brazil
Araucaria	Unknown	Brazil
Hu39694	Unknown	Argentina

HFRS=hemorrhagic fever with renal syndrome
HCPS= hantavirus cardiopulmonary syndrome
The table is adapted from two articles (Khaiboullina et al. 2005, Jonsson et al. 2010).

2.1.2 Epidemiology

Only around 2,000 HCPS cases have been reported world wide so far, with approximately 300 people being affected annually (Muranyi et al. 2005, Jonsson et al. 2010). At the same time, HFRS affects approximately 150,000-200,000 people every year (Muranyi et al. 2005, Jonsson et al. 2010). More than half of the cases occur in China, where HTNV and SEOV viruses cause HFRS (Jonsson et al. 2010). In Europe, PUUV causes most HFRS cases (Heyman and Vaheri 2008). A minority of HFRS cases in Europe are caused by DOBV in the Balkans and SAAV (Heyman and Vaheri 2008).

Finnish PUUV infections account for approximately 70 % of all European HFRS cases (Heyman and Vaheri 2008). During recent years, the annual number of serological diagnoses of PUUV infection has been approximately 1,000-3,000 in Finland (THL 2012). The annual incidence has an increasing trend, with an average annual incidence of 31/100,000 (Makary et al. 2010). The average PUUV seroprevalence in Finnish population is 5 %, implying that many infections remain undiagnosed or present as subclinical (Brummer-Korvenkontio et al. 1999). Earlier, it had been observed that outbreaks occurred usually every 3-4 years. However, a recent study detected that since 1998, two consecutive years with high epidemic peaks were followed by one year with a low epidemic peak (Makary et al. 2010). In addition, the incidence varies widely by season. The epidemic usually starts in late summer, with an increasing incidence in late autumn or early winter (Makary et al. 2010). During spring, the incidence is at its lowest. The age groups 34-49 years as well as 50-64 years have the highest incidence and the majority of the patients (62 %) are males (Makary et al. 2010). Other countries besides Finland, where more than one thousand PUUV cases in total have been recorded, include Sweden, Norway, Belgium, France, and Germany (Heyman and Vaheri 2008). However, some countries, such as Estonia, have not reported their PUUV cases until recently.

2.2 Hantaviral clinical manifestations

2.2.1 Hemorrhagic fever with renal syndrome

The clinical picture of HFRS varies from asymptomatic to fatal. In PUUV, SAAV and SEOV infections, the mortality rate is low, while in HFRS cases caused by HTNV or DOBV, it varies from 3 to 16 % (Kanerva et al. 1998a, Avsic-Zupanc et al. 1999, Peters et al. 1999, Vapalahti et al. 2003). The disease can be divided into five phases: febrile, hypotensive, oliguric, polyuric, and convalescence (Kanerva et al. 1998a, Peters et al. 1999, Jonsson et al. 2010).

The disease starts with a sudden onset of high fever, followed by headache, back and abdominal pains and nausea (Kanerva et al. 1998a, Peters et al. 1999). Additional findings during the febrile phase include photophobia, myopia, dizziness, flushing of the face, periorbital edema, and conjunctival infection (Kanerva et al. 1998a, Peters et al. 1999). The febrile phase lasts for 3-5 days, and at the end of this phase, hypotension may develop rapidly leading, in severe cases, to shock and cardiovascular collapse (Kanerva et al. 1998a, Peters et al. 1999).

After the febrile and hypotensive phases, the oliguric phase begins, lasting for 1-16 days (Jonsson et al. 2010). Hemodialysis treatment is needed for approximately 20 % of patients with SEOV infection and for 40 % of patients with HTNV infection, whereas among NE patients the need for hemodialysis is only up to 6 % (Mustonen et al. 1994a, Jonsson et al. 2010). Petecchiae are common and also severe internal bleedings can be seen, especially in HTNV infection (Kanerva et al. 1998a, Jonsson et al. 2010). Furthermore, disseminated intravascular coagulopathy (DIC) is found in 20 % of HTNV patients (Kanerva et al. 1998a, Peters et al. 1999). Typical laboratory findings include thrombocytopenia, leukocytosis with a left shift, increased hematocrit due to vascular leakage, elevated serum creatinine level, elevated liver enzymes, hypoproteinemia, as well as proteinuria and hematuria (Kanerva et al. 1998a, Vapalahti et al. 2003, Jonsson et al. 2010).

The oliguric phase accounts for approximately 50 % of all HFRS-related deaths (Jonsson et al. 2010). In most cases, mortality caused by HFRS is due to complications from renal insufficiency, shock, or hemorrhages (Jonsson et al. 2010). After the polyuric phase has started, recovery is the rule (Kanerva et al. 1998a).

2.2.2 Nephropathia epidemica

2.2.2.1 Clinical characteristics

In acute NE, the incubation period varies from 1 to 8 weeks (Settergren et al. 1989). The disease starts with sudden high fever and headache, followed by nausea, vomiting, abdominal pains, and backache (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010). Myalgia and visual disturbances are also common. The ocular symptoms have multifactorial origin, they are partly due to a myopic shift, but also extraocular mechanisms may be involved (Hautala et al. 2011a). Distinguishing the typical five phases of HFRS (febrile, hypotensive, oliguric, polyuric, and convalescent) may be difficult and they are not always present due to the relative mildness of the disease.

Serious hemorrhagic complications are rare in NE. However, mild bleeding manifestations occur, such as conjunctival or retinal bleeding, petechiae, macroscopic hematuria, melena, hematemesis, epistaxis and bleeding from puncture sites (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a). Some hemorrhagic manifestation has been reported in 10-37 % of patients and epistaxis in 11-28 % of patients (Lähdevirta 1971, Settergren et al. 1989). In a Finnish study with 10 patients, mild gastrointestinal bleeding was demonstrated by gastroscopy in all of the patients studied (Nuutinen et al. 1992). Furthermore, there are case reports of hypophyseal hemorrhages, as well as, in rare fatal cases, hemorrhages of other organs (Valtonen et al. 1995, Hautala et al. 2002).

Central nervous system (CNS)-related symptoms are usual in NE. Typical manifestations include headache, insomnia, as well as somnolence, dizziness, restlessness, anxiety, and amnesia (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010). In a recent study, 58 patients with PUUV infection were studied and 87 % suffered symptoms suggestive of CNS involvement (Hautala et al. 2010). In this study, also cerebrospinal fluid was studied and, in half of the samples, it proved positive for PUUV immunoglobulin (Ig)M, elevated protein level, or leukocyte count. Magnetic resonance imaging (MRI) revealed pituitary hemorrhage in 2/58 patients (Hautala et al. 2010). Young male patients have been shown to be at elevated risk for serious CNS complications during NE (Hautala et al. 2011b).

There are case reports of hypopituitarism during acute NE linked to pituitary hemorrhages or other pituitary abnormalities in MRI (Hautala et al. 2002, Hautala et al. 2010, Hautala et al. 2011b). In a recent study, hormonal deficiencies were examined in 54 patients (Mäkelä et al. 2010). It was revealed that 56 % of patients had abnormalities of the gonadal and/or thyroid axis during the acute infection. The acute hormonal alterations of central origin were associated with the severity of renal impairment and the degree of inflammation. The endothelial damage and increased vascular permeability during the acute infection, as well as the tight interaction between the immune and endocrine systems could be involved in the pathogenesis of the hormonal defects (Mäkelä et al. 2010).

2.2.2.2 Renal involvement

Renal involvement is manifested by transient proteinuria, microscopic hematuria, and renal function impairment, which is demonstrated as oliguria and a rise in serum creatinine level (Lähdevirta 1971). Oliguria is then followed by polyuria and a spontaneous recovery (Lähdevirta 1971). Transient hemodialysis treatment is needed by up to 6 % of hospital-treated patients (Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010).

The characteristic histopathologic renal finding is acute tubulointerstitial nephritis. Interstitial edema, inflammatory cell infiltrations, as well as tubular epithelial and luminal alterations are seen (Mustonen et al. 1994b). The infiltrating cells include plasma cells, monocytes, macrophages, and lymphocytes, as well as polymorphonuclear cells, mainly eosinophils and neutrophils (Mustonen et al. 1994b, Temonen et al. 1996). CD8+ T cells predominate the lymphocytic infiltrate (Temonen et al. 1996). Also interstitial hemorrhages are seen in 20-60 % of biopsies (Collan et al. 1991, Mustonen et al. 1994b). In immunofluorescence (IF) analysis, deposits of IgG, IgM, complement component C3 and fibrinogen have been found along the tubular basement membrane in about half of the cases (Collan et al. 1991). Also weak glomerular mesangial alterations are present in 25 % of the cases (Mustonen et al. 1994b). Furthermore, IF has revealed glomerular deposits of IgG, IgM, IgA and complement components C3 and C1q (Collan et al. 1991, Mustonen et al. 1994b). However, in a Finnish study with 86 patients, the glomerular IF

finding was normal in 43 % of the biopsies (Mustonen et al. 1994b). Tubular, interstitial and glomerular histologic lesions have been associated with the clinical severity of renal failure (Mustonen et al. 1994b). However, the glomerular alterations have not related to the amount of urine protein excretion (Mustonen et al. 1994b).

2.2.2.3 Cardiological findings

In a Croatian study of 79 patients with HFRS, electrocardiography (ECG) alterations were present in 38 % of the patients and three patients were diagnosed to have myocarditis (Puljiz et al. 2005). All ECG changes were transient in this study. There are also some case reports of myocarditis in patients with PUUV infection (Lähdevirta 1971, Mustonen et al. 1994a, Valtonen et al. 1995). In a Finnish study with 70 PUUV-infected patients, ECG changes were observed in 57 % of patients (Mäkelä et al. 2009). Moreover, in this study, echocardiography (ECHO) showed impaired left ventricular contraction in six patients and mild pericardial effusion in one patient. All ECG and ECHO findings returned to normal. Acute renal failure with fluid retention, abnormal plasma electrolyte levels, fever, and cytokine release could be the possible pathogenetic mechanisms for the myocardial involvement (Mäkelä et al. 2009). However, no differences were found in the clinical or laboratory findings between patients with and without ECG or ECHO changes. Thus, the pathogenesis of the cardiac involvement in NE is unclear.

2.2.2.4 Laboratory findings

Thrombocytopenia is seen in 57-75 % of patients with acute PUUV infection (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010). In a Finnish study with 126 patients, the mean minimum platelet count reduced clearly and was 117 $x10^9$/l, while the lowest platelet count was 10 $x10^9$/l (Mustonen et al. 1994a). In a Swedish study with 74 patients, the median platelet count was 96 $x10^9$/l (Settergren et al. 1989). The mechanisms behind this phenomenon remain to be clarified. DIC promoted by vascular injury has been suggested as a possible cause of thrombocytopenia in NE (Cosgriff 1991). DIC has been reported in 5-26 %

of PUUV-infected patients (Settergren et al. 1989, Laine et al. 2010). In a Finnish study with 19 patients with NE, thrombocytopenia was detected to associate with decreased natural anticoagulants, shortened thrombin time and enhanced fibrinolysis, but not with the degree of renal insufficiency (Laine et al. 2010). It is suggested that the interaction of platelets with endothelium, their activation and P-selectin expression could provide mechanisms of thrombocytopenia during hantavirus infection. Furthermore, enhanced platelet adhesion and activation could result in platelet consumption and thrombocytopenia (Laine et al. 2011).

Anemia is present in 33-50 % of patients with NE (Lähdevirta 1971, Mustonen et al. 1994a). It is probably due to the infection itself, as well as renal insufficiency and blood dilution during the oliguric phase, although hemorrhagic manifestations can also play a role. Hemoconcentration caused by increased capillary permeability on the other hand, can cause increased hemoglobin levels, which has been detected in 12-52 % of patients with NE (Lähdevirta 1971, Settergren et al. 1989). Leukocytosis has been reported in 36-57 % of patients and elevated CRP level in almost all of the patients (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010). However, CRP level has ranged from 0 mg/l to 295 mg/l (Settergren et al. 1989, Mustonen et al. 1994a).

In most hospital-treated patients (85-96 %) with acute PUUV infection, serum creatinine level is elevated (Lähdevirta 1971, Settergren et al. 1989, Braun et al. 2010). In a Finnish study with 126 patients, the mean creatinine value was 439 μmol/l while in a Swedish study with 74 patients, the median creatinine value was 386 μmol/l and 35 % of patients had creatinine >500 μmol/l (Settergren et al. 1989, Mustonen et al. 1994a). In urinalysis, proteinuria is the most common finding, detected in 82-100 % of patients, and it is in the nephrotic range in 25-34 % of patients (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Mäkelä et al. 2004, Braun et al. 2010). Microscopic hematuria is present in 58-85 % of patients (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010).

Other often recorded laboratory findings in NE include elevated liver enzymes, transient electrolyte abnormalities, such as hypocalcemia, hyponatremia, hyperphosphatemia, hypokalemia and hyperkalemia, as well as hypoproteinemia due to hypoalbuminemia (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a).

2.2.2.5 Radiological findings

Abnormal findings on chest radiographs have been reported in 16-35 % of patients with NE (Lähdevirta 1971, Mustonen et al. 1994a, Kanerva et al. 1996, Paakkala et al. 2004b). Pleural effusion, atelectasis, and interstitial infiltration are the most common X-ray findings (Kanerva et al. 1996, Paakkala et al. 2004b). Radiological pulmonary manifestations have been associated with the degree of renal insufficiency and fluid volume overload, as well as with high blood pressure, leukocytosis and thrombocytopenia (Kanerva et al. 1996, Paakkala et al. 2004b). In a Swedish study, 19 patients were studied with pulmonary computed tomography (CT) and infiltrates or pleural effusions were seen in 10 (53 %) patients (Linderholm et al. 1992). A recent Finnish study with 13 patients showed that, when examined with high-resolution pulmonary CT, almost every patient (12/13) showed lung parenchymal abnormalities (Paakkala et al. 2011).

Renal ultrasound (US) findings in PUUV-infected patients have been analyzed in three studies (Paakkala et al. 2002, Paakkala et al. 2004a, Paakkala et al. 2004b). When renal US was performed on 23 patients, the findings were abnormal in every case (Paakkala et al. 2002). The resistive index was abnormal in 12/23 patients and fluid collections were found in 13/23 patients. Furthermore, the severity of the findings was associated with fluid volume overload and the degree of renal insufficiency (Paakkala et al. 2002, Paakkala et al. 2004b). The kidneys were also examined by renal MRI as well as magnetic resonance renography (MRR) in 20 Finnish NE patients (Paakkala et al. 2005, Paakkala et al. 2006). Renal MRI changes occurred in every patient and the severity of the findings in MRI was mildly associated with severe renal insufficiency and fluid volume overload, as well as with high blood pressure, inflammation, and thrombocytopenia (Paakkala et al. 2005). Measurable functional MRR findings, in turn, were recorded in 14/20 patients and the severity of these findings had mild association with the degree of renal insufficiency and fluid volume overload (Paakkala et al. 2006).

2.2.2.6 Diagnosis

The diagnosis of acute PUUV infection is based on the clinical picture and is serologically confirmed. It is based on an IgM-capture enzyme immunoassay test

and baculovirus-expressed PUUV full-length N protein (Vapalahti et al. 1996, Vaheri et al. 2008). A rapid immunogromatographic PUUV IgM test is also available (Hujakka et al. 2001). Antibodies are present usually in the first serum sample taken, but in 2-4 % of PUUV infections, seroconversion may take up to five days after the onset of the disease (Kallio-Kokko et al. 1998).

2.2.2.7 Treatment and prevention

The treatment of NE consists of supportive care with careful monitoring and management of fluid and electrolyte balance, diuresis, and respiration, as well as pain relief. In addition to hemodialysis therapy, ventilation support may be needed.

There is no specific therapy available for NE. The antiviral drug ribavirin has been studied in the treatment of HFRS in China, in a prospective, double-blind and placebo-controlled trial (Huggins et al. 1991). This study showed a sevenfold decrease in mortality risk with intravenous ribavirin therapy. Furthermore, oliguria and hemorrhages were less common in the ribavirin group (Huggins et al. 1991). Another study in HFRS patients were carried out in Korea and it suggested that ribavirin therapy may decrease renal complications (Rusnak et al. 2009). However, there are no studies regarding PUUV-infected patients and ribavirin therapy.

In the context of prevention, avoiding the exposure to rodents and their excreta is of importance. The most important risk factors for contracting PUUV infection are smoking and living in buildings with holes allowing rodents to enter (Vapalahti et al. 2010). Vaccines have been developed against viruses causing HFRS in Asia, but there are no vaccines available against DOBV or PUUV (Schmaljohn 2009). However, there are recent promising results from a phase 1 study concerning HTNV and PUUV DNA vaccines tested as single or in combination in three groups of nine volunteers (Boudreau et al. 2012).

2.2.2.8 Prognosis

The natural course of NE is usually favorable and the outcome is spontaneous recovery. However, in rare cases, the disease can be fatal. The reported mortality in Finland is 0.08 % (Makary et al. 2010).

There are several studies concerning the long-term outcome of PUUV infection. In two earlier Finnish studies, 20 and nine patients were studied 1-7 years and 4-5 years after acute NE, respectively (Lähdevirta 1971, Lähdevirta et al. 1978). In the first study, it was detected that five patients had slightly reduced creatinine clearance and the renal concentration capacity was decreased in eight patients (Lähdevirta 1971). In the second study, creatinine clearance was normal in all of the patients, but five patients had slightly depressed tubular function (Lähdevirta et al. 1978). In two more recent Finnish studies with 46 and 37 patients with previous NE and 38 healthy seronegative controls, it was revealed that 5-6 years after NE, the patients had higher glomerular filtration rate, greater urinary protein excretion and higher systolic blood pressure compared to the controls (Mäkelä et al. 2000, Miettinen et al. 2009). Thirty-six patients who participated in the first study were also examined 10 years after acute NE (Miettinen et al. 2006). It was revealed that the glomerular hyperfiltration and proteinuria detected at five years after the acute disease had disappeared. The prevalence of hypertension was also no longer statistically significantly higher than in the controls. However, the possibility remained that NE may dispose some patients to the development of hypertension. Otherwise, the long term prognosis of NE is favorable (Miettinen et al. 2006). Finally, another study revealed that the clinical severity of acute PUUV infection does not predict the long term outcome with reference to renal function, blood pressure or 24-hour urinary protein excretion (Miettinen et al. 2010).

Furthermore, the prognosis of the hormonal deficiencies detected during acute NE has been studied (Mäkelä et al. 2010). Thirty patients out of 54 had hormonal alterations during the acute phase. After a median follow-up of five years, nine patients (17 %) were diagnosed with a chronic hormonal deficit. Hypopituitarism, primary hypothyroidism and chronic testicular failure were each diagnosed in five patients. The occurrence of these long-term hormonal defects was not associated with the severity of the acute infection (Mäkelä et al. 2010).

2.2.3 Hantavirus cardiopulmonary syndrome

In contrast to HFRS, HCPS is typically characterized by cardiopulmonary dysfunction instead of hemorrhages and renal failure (Kanerva et al. 1998a, Peters et

al. 1999, Jonsson et al. 2010, Simpson et al. 2010). The severity of this dysfunction can range from mild hypoxemia with stable hemodynamics to rapidly progressive respiratory failure with cardiogenic shock (Peters et al. 1999). The disease can be divided into four clinical phases: prodrome, pulmonary edema and shock, diuresis, and convalescence (Simpson et al. 2010). After an incubation period of 1-6 weeks, the disease starts with fever, chills, myalgia, headache, and gastrointestinal symptoms (Kanerva et al. 1998a, Peters et al. 1999, Jonsson et al. 2010, Simpson et al. 2010). Then, after 3-6 days, progressive cough, tachypnea, tachycardia, and hypotension develop, leading to respiratory decompensation, pulmonary edema and shock (Kanerva et al. 1998a, Simpson et al. 2010). Mortality most commonly occurs within the first 24 hours of this phase (Simpson et al. 2010). Again, after 3-6 days, surviving patients enter the diuretic phase with rapid resolution of respiratory and hemodynamic abnormalities (Simpson et al. 2010).

Typical laboratory findings in HCPS are thrombocytopenia, leukocytosis with a left shift, and circulating immunoblastoid lymphocytes (Peters et al. 1999, Simpson et al. 2010). Although thrombocytopenia is present in 79 % of patients, hemorrhages are rare (Simpson et al. 2010). Hemoconcentration due to capillary leakage, elevated liver enzymes and lactate dehydrogenase, hypoalbuminemia, as well as proteinuria are also common findings (Simpson et al. 2010). Although 20-48 % of patients present with elevated creatinine levels, severe renal failure is uncommon (Peters et al. 1999, Simpson et al. 2010).

Chest radiographic abnormalities are present in most patients on admission and the typical finding is interstitial pulmonary edema (Kanerva et al. 1998a, Peters et al. 1999, Simpson et al. 2010). Two thirds of the patients subsequently develop alveolar edema, which is typically bibasilar and perihilar (Kanerva et al. 1998a, Peters et al. 1999). Furthermore, pleural effusion develops in all patients as the disease progresses (Simpson et al. 2010). The heart size remains normal, but ECHO reveals moderately to severely depressed left ventricular systolic function (Peters et al. 1999). Death, if it occurs, is caused by progressive myocardial insufficiency (Simpson et al. 2010).

The mortality of HCPS is high, 35-60 % (Jonsson et al. 2010, Simpson et al. 2010). The surviving patients take typically a few months to convalesce, but it can take as long as two years to fully recover (Simpson et al. 2010).

Recently, growing evidence has showed that there are similarities in the clinical picture of HFRS and HCPS and the symptoms overlap to some extent (Rasmuson et al. 2011a, Clement et al. 2012).

2.3 Pathogenesis and immunology in hantaviral infections

2.3.1 Increased capillary permeability

Pathological changes in both HFRS and HCPS are characterized by an increased capillary permeability in the affected organs and endothelial cells are considered the primary targets of hantavirus infection (Cosgriff 1991, Zaki et al. 1995, Kanerva et al. 1998a). Increased capillary permeability and vascular leakage explain many signs and symptoms in HFRS and HCPS, such as hypotension and shock, abdominal pains and retroperitoneal edema, as well as pleural effusion and pulmonary edema (Cosgriff 1991, Kanerva et al. 1998a). The exact pathogenetic mechanisms behind this central phenomenon in hantavirus infections are currently not completely understood.

2.3.2 Apoptosis

Apoptosis is a genetically controlled cell death process playing an important role in physiological conditions, such as multicellular organism development and tissue regeneration, as well as in some pathological conditions including inflammation and infection (Strasser et al. 2000). Hantaviruses have been considered noncytopathic. Endothelial cells, the primary targets in naturally acquired hantavirus infections, are infected *in vitro* with no cytopathic effects (Yanagihara and Silverman 1990). Human cell lines infected with PUUV have also shown no cytopathic effects (Temonen et al. 1993). However, under certain conditions Tula hantavirus induces apoptosis in cultured Vero E6 (green monkey kidney) cells, a commonly used cell line in hantavirus infections (Li et al. 2004, Li et al. 2005). Furthermore, two studies have shown that hantavirus infection is able to cause apoptosis in Vero E6 or human

embryonic kidney cells (Kang et al. 1999, Markotic et al. 2003). In addition, apoptosis has been detected in lymphocytes during HFRS (Akhmatova et al. 2003)(cited in (Li et al. 2005)). Finally, a Swedish study with 18 PUUV-infected patients showed that serum level of the epithelial cell apoptosis marker, caspase-cleaved cytokeratin-18, is increased during the acute infection indicating apoptosis of epithelial cells (Klingström et al. 2006). The tissue damage is suggested to be due to immunopathogenic mechanisms.

2.3.3 Integrins and vascular endothelial growth factor

The cellular entry of pathogenic hantaviruses is mediated by β_3-integrins (Gavrilovskaya et al. 1998, Gavrilovskaya et al. 1999). Integrins are heterodimeric surface receptors on endothelial cells and platelets mediating cell-to-cell adhesion, cell migration, extracellular matrix protein recognition, and platelet aggregation. Integrins are composed of α and β subunits (Albelda and Buck 1990). β_3-integrins have an important role in regulating vascular integrity, endothelial cell permeability, and platelet functions (Mackow and Gavrilovskaya 2009). Pathogenic hantaviruses may inhibit these functions, thus interfering with the endothelial permeability. Supporting this assumption, it has been demonstrated that pathogenic hantaviruses inhibit β_3-integrin directed endothelial cell migration, whereas non-pathogenic hantaviruses do not (Gavrilovskaya et al. 2002). Further, the surface density of platelet β_3-integrin has been demonstrated to correlate with disease severity in HTNV infection (Liu et al. 2008).

Vascular endothelial growth factor (VEGF) is expressed on angiogenic endothelium and is able to induce vascular permeability (Dvorak 2006). β_3-integrins regulate vascular permeability through effects on VEGF (Gavrilovskaya et al. 2008). β_3-integrin and VEGF receptor-2 (VEGFR2) form a functional complex and interact with each other (Wang et al. 2012). It has been demonstrated that pathogenic hantaviruses enhance the permeability of endothelial cells in response to VEGF, while non-pathogenic hantaviruses have no effect on endothelial cells (Gavrilovskaya et al. 2008, Wang et al. 2012). This occurs concurrently with inhibition of β3-integrin functions (Gavrilovskaya et al. 2008). Further, hantavirus-directed permeability has been inhibited by antibodies against VEGF2

(Gavrilovskaya et al. 2008, Gorbunova et al. 2011). This finding may offer a therapeutic possibility in hantavirus infections.

2.3.4 T lymphocytes

The cytotoxic CD8+ T lymphocytes specific for hantavirus are assumed to play an important role in the pathogenesis of hantaviral infections (Terajima et al. 2007). On the other hand, T cells have an essential role in the clearance of the virus infection (Terajima and Ennis 2011).

At the onset of HFRS and HCPS, increased amounts of circulating CD8+ T cells are observed (Huang et al. 1994, Ennis et al. 1997, Kilpatrick et al. 2004). A recent Swedish study showed that, in NE, a primary effector CD8+ T cell response develops rapidly after virus infection peaking within two weeks after the beginning of symptoms (Lindgren et al. 2011). In HTNV infection, also a decreased CD4+ helper cell/CD8+ ratio has been demonstrated (Huang et al. 1994). In addition, CD8+ T lymphocytes predominate the cell infiltrate in the kidneys during the acute phase of NE as well as in the lungs in lethal HCPS cases (Zaki et al. 1995, Temonen et al. 1996). Bronchoalvelolar lavage fluid from patients with NE has also been shown to contain higher amount of CD8+ T cells and natural killer (NK) cells compared to healthy controls (Linderholm et al. 1993). Endobronchial mucosal biopsies from patients with NE have revealed increased numbers of both CD8+ and CD4+ T cells (Rasmuson et al. 2011b). These findings indicate a local immune response in the lungs. A recent study detected that urine type 2 cytokine-specific transcription factor (GATA-3) necessary for the generation of type 2 T cells is an independent risk factor for severe PUUV-induced acute kidney injury either reflecting enhanced type 2 T cell responses or kidney injury (Libraty et al. 2012). Furthermore, a rapid expansion and long-term persistence of elevated NK cells in PUUV infection has also been reported recently (Björkström et al. 2011).

The virus-specific CD8+ memory T cell population has been demonstrated to develop during the convalescent phase of NE (Tuuminen et al. 2007). Furthermore, the memory T cells have been shown to persist thereafter for several years as well after PUUV as ANDV infection (Van Epps et al. 2002, Manigold et al. 2010). These

persisting memory T cells may play a role in the long-lasting immunity after a hantavirus infection.

It has been suggested that virus-specific cytotoxic T cells play an important role in the development of endothelial cell dysfunction and capillary leakage in HFRS and HCPS (Terajima et al. 2007). Supporting this idea, it has been shown that hantavirus-specific cytotoxic T cells recognize and increase the permeability of human endothelial cells infected with SNV (Hayasaka et al. 2007). Furthermore, the frequency of circulating virus-specific CD8+ T cells has been demonstrated to associate with the severity of HCPS (Kilpatrick et al. 2004). CD4+ regulatory T-cells (Tregs), in turn, have been shown to be reduced in HFRS compared to healthy controls and correlate negatively with the severity of the disease (Zhu et al. 2009). Inefficient control of effector T cells by Tregs may contribute to the pathogenesis of hantavirus infection. A Chinese study reported that the frequency of HTNV-specific T cells was lower in patients with severe disease (Wang et al. 2009a). However, in this study, the frequency of CD4+ and CD8+ T cells was combined.

2.3.5 Cytokines

Cytokines are mediators of information between cells. They are produced by a number of different cell types, such as monocytes, macrophages, and lymphocytes, in response to inflammatory signals and participate in the regulation of the inflammatory response. Cytokines can be functionally divided into pro-inflammatory (such as IL-1, IL-6, TNF-α, IL-2, IL-12) and anti-inflammatory (such as transforming growth factor (TGF)-β, IL-1Ra, IL-10) cytokines. In addition to acute phase protein induction, cytokines, especially TNF-α, IL-1, and IL-6, are mediators responsible for fever and septic shock (Akira et al. 1990, Tracey and Cerami 1994). The cytokine production occuring during hantaviral infection may be one of the major causes of the symptoms in HFRS and HCPS and they are thought to play an important role in the vascular leakage observed in these diseases. TNF-α is known to be able to increase vascular permeability (Tracey and Cerami 1994).

Increased cytokine levels have been found in plasma, urine and tissues of patients with hantaviral infection. In HFRS caused by HTNV, elevated levels of IFN-α and IFN-γ were found in sera of 110 patients of the Korean war (Krakauer et al. 1994).

Further, IL-1β was detectable, whereas in healthy controls it was not (Krakauer et al. 1995). A Swedish study with 15 patients with NE showed IL-6 and TNF-α concentrations to be elevated in all and IL-10 concentrations in most patients in the acute phase (Linderholm et al. 1996). Maximum levels of TNF-α and IL-6 also correlated positively with the maximum level of serum creatinine and TNF-α also inversely with mean blood pressure. A Finnish study conducted with 19 PUUV-infected patients showed soluble IL-2 receptor (sIL-2R), IL-6, and IL-8 concentrations to be elevated. There was also an inverse correlation between the mean arterial pressure and sIL-2R as well as between minimum platelet count and sIL-2R and IL-6 (Takala et al. 2000). However, no correlation between serum creatinine and cytokine levels was found in this study.

A Finnish study with 70 PUUV-infected patients found plasma levels and urinary excretion of IL-6 to be increased. However, there was no correlation between plasma and urinary levels indicating possible local production of IL-6 in the kidneys (Mäkelä et al. 2004). Urinary excretion of IL-6 correlated with urinary albumin, IgG and protein excretions, but not with serum creatinine levels. In another Finnish study, TNF-α, TGF-β, and platelet-derived growth factor expression was detected to be increased in the kidneys of PUUV-infected patients in the peritubular area of the distal nephron (Temonen et al. 1996). In a study carried out in the United States, cytokine-producing cells were detected in the lungs, kidneys, liver, and spleen of patients with fatal HCPS (Mori et al. 1999). The number of cytokine-producing cells in the lungs was higher than in the kidneys and in the liver suggesting that local cytokine production may play an important role in the pathogenesis of HCPS.

Some recent studies have provided more information concerning cytokines in hantaviral infections. A Swedish study compared cytokine levels between 19 male and 20 female patients with NE (Klingström et al. 2008). Interestingly, the females showed higher plasma levels of IL-9, fibroblast growth factor 2, and granulocyte-macrophage colony-stimulating factor and lower levels of IL-8 and IFN-γ-induced protein 10 in the acute phase of the disease as compared to the males. Thus, PUUV infection may induce sex-dependent differences in the innate immune responses in humans, which may contribute to the higher incidence of NE among males.

A Slovenian study was carried out with 61 patients with PUUV infection and 52 patients with DOBV infection (Saksida et al. 2011). Increased levels of IL-10, IFN-γ, and TNF-α were found in almost all of the serum samples. The concentrations

were higher in patients infected with DOBV than PUUV. Furthermore, the levels of IL-10 and TNF-α were higher in patients with a more severe clinical course of DOBV infection. However, PUUV-infected patients presented no differences in cytokine concentrations according to disease severity, but showed higher IL-12 levels than DOBV-infected patients. The authors suggested that the imbalance in the production of pro-inflammatory and regulatory cytokines may be associated with the disease severity of hantaviral infection (Saksida et al. 2011).

This hypothesis is supported by the results of a study with 21 HCPS patients and 21 controls carried out in Brazil (Borges et al. 2008). In this study, the levels of pro-inflammatory IL-6 and TNF-α as well IFN-γ were found to be elevated but the level of an anti-inflammatory cytokine, TGF-β, was reduced. The levels of the pro-inflammatory cytokines correlated with disease severity and, in fatal cases, very high IL-6 levels were seen. Finally, a German study with 64 patients with acute NE support the idea of imbalance in cytokine production (Sadeghi et al. 2011). Significantly elevated levels of IL-2, IL-6, IL-8, TGF-β1, and TNF-α were detected. Furthermore, disease severity characterized by elevated creatinine and low platelet counts correlated with high pro-inflammatory IL-6 and TNF-α levels but low anti-inflammatory TGF-β1 levels. Also the cytokine levels in the early and late phases of the disease were compared. The levels of the pro-inflammatory cytokines decreased, whereas TGF-β1 levels increased. The authors conclude that possibly delayed induction of the protective immune mechanism to downregulate the early pro-inflammatory immune response contributes to the pathogenesis of human hantaviral infection.

2.3.6 Host genetic factors

The clinical course of hantaviral infections is influenced by host-related factors. Several studies have been carried out in relation to host genetics in NE. Human leukocyte antigens (HLA) are major cell surface antigens, whose role is to present pathogen-derived antigens to T cells and to initiate adaptive immune responses (Klein and Sato 2000). In a Finnish study with 74 patients, HLA alleles B8, C4A*Q0, and DRB1*0301 associated with the most severe form of the disease (Mustonen et al. 1996). Furthermore, all patients suffering from shock and most

patients requiring dialysis treatment were positive for HLA B8 allele. On the contrary, HLA B27 was shown to be less frequent in patients with NE than in the general population and it was associated with a mild form of the disease (Mustonen et al. 1998). In 39 Finnish pediatric patients, no significant differences in the clinical picture with and without HLA B8-DRB1*03 haplotype were found (Mustonen et al. 2004). However, this haplotype was detected in a significantly higher proportion of patients than in the general population.

Polymorphism at position -308 of the TNF-α gene promoter region was studied in 59 Finnish patients and 40 controls (Kanerva et al. 1998b). TNF2, a high-producing genotype of TNF-α, was found to be more frequent in hospitalized NE patients as compared to controls. Yet another Finnish study showed that the clinical course of NE is more severe in TNF2 carriers than non-carriers (Mäkelä et al. 2001). However, a study on TNF-α gene promoter polymorphism at position -238 in 36 Belgian patients showed that the low producer genotype was associated with a more severe clinical course of NE (Maes et al. 2006). The discrepancy between these results may be explained by the findings of 116 patients with NE in a Finnish study, where the TNFα(-308) showed unlikely to be of marked significance to the outcome of NE (Mäkelä et al. 2002). The association of TNF2 allele with severe NE is probably due to strong linkage with HLA-B8-DR3 haplotype. Thus, TNF2 is not an independent risk factor for severe NE, but a passive component in the extended haplotype. Moreover, a Finnish study with 87 NE patients and 400 blood donors as controls indicated that NE patients were more often IL-1receptor antagonist-2 allele and IL-1β-2 allele negative than the seronegative controls (Mäkelä et al. 2001).

Host genetic factors in association with chest radiography findings have been studied in 114 Finnish patients with NE (Paakkala et al. 2008). Both the presence and severity of abnormal NE-related radiography findings associated with the B8, DR3, and TNF2 alleles. Pleural effusion, a sign of increased capillary permeability, showed the strongest association with these genetic factors. The association of HLA haplotype with CNS-related symptoms has been studied in 58 Finnish patients with NE (Hautala et al. 2010). A significant negative correlation between cerebrospinal fluid inflammation and DR15(2)-DQ6 haplotype was found, indicating that host genetics may have a role in CNS involvement. HLA-B, HLA-DRB1, TNF-α(-308) and IL-6(-174) alleles were studied in 43 patients six years after NE (Miettinen et al.

2010). The genetic factors determined did not predict the long term outcome of the patients.

Studies concerning other hantaviruses than PUUV and host genetic factors are not abundant. In Chinese patients with HFRS caused by HTNV, HLA-DRB1*09 and HLA-B*46-DRB1*09 haplotypes were significantly more frequent than in controls in a study with 77 patients and 83 healthy controls (Wang et al. 2009b). A Brazilian study with 26 HCPS patients and 96 individuals with hantavirus seroconversion found TNF2 allele more frequent among the patients than in individuals with positive serology without a history of HCPS (Borges et al. 2010). A Slovenian study examined HLA haplotypes in 88 PUUV-infected and 72 DOBV-infected patients (Korva et al. 2011). PUUV-infected patients, especially with a severe form of the disease, showed to have more frequently HLA-DRB1*13 haplotype than DOBV-infected patients. HLA-B*07, in turn, showed to have a possible protective role in PUUV infection. Furthermore, DOBV-infected patients had a significantly higher frequency of HLA-B*35 than PUUV-infected patients. Thus, different hantaviruses may be presented differently through the same HLA molecules.

2.3.7 Complement system

The complement system has three major pathways: the classical, alternative, and the lectin-dependent pathway. These pathways are activated differently, but they all converge at the point of cleavage of complement component C3 (Walport 2001). The end-product of the complement cascade is the cytolytic membrane-attack complex, which is formed by sequential assembly of the complement components C5b, C6, C7, and C9 to a target cell membrane (Walport 2001). If the complexes are formed without a target membrane in a fluid phase, C5b-9 binds to S-protein or clusterin, and a non-lytic soluble SC5b-9 terminal complex is formed (Podack and Muller-Eberhard 1979).

The activation of the complement system in acute PUUV infection has been analyzed in two Finnish studies. In the first study, 25 patients with acute NE were examined (Paakkala et al. 2000). Complement activation was observed in 23 (92 %) patients. In 10 patients, the complement system was activated mainly through the

alternative route, in six, mainly through the classical route and, in five, through both the alternative and the classical route. Furthermore, the classical pathway activation was associated with a severe clinical course of NE (Paakkala et al. 2000). A recent study with 61 patients with acute NE analyzed the levels of SC5b-9, C3, and C4 (Sane et al. 2011). It was found that the alternative pathway of the complement system was activated during the acute phase of the infection, as evidenced by increased levels of SC5b-9 and decreased levels of C3. Apparently, the level of complement activation correlated with the severity of the disease. It is further suggested that the complement activation may contribute to the pathogenesis of acute NE by contributing to the development of vascular leakage (Sane et al. 2011).

2.3.8 Humoral immunity

Both HFRS and HCPS induce high levels of virus-specific IgM directed against the N protein as well as Gn and Gc glycoproteins (Groen et al. 1992, Lundkvist et al. 1993, Bostik et al. 2000). The N protein, however, has been demonstrated to be the major antigenic protein in PUUV infection (Lundkvist et al. 1993). Hantavirus-specific IgM antibodies are positive early after the onset of the disease, remain detectable during the convalescent phase, and usually become undetectable 2-5 months after the infection (Lundkvist et al. 1993, Elgh et al. 1998).

Hantavirus-specific IgG antibodies also appear during the acute phase, but an increase in IgG levels is seen during the early convalescent phase (Groen et al. 1992, Lundkvist et al. 1993). The acute phase IgG response is mainly directed towards the N protein, while IgG antibodies directed against the glycoproteins appear later in the early convalescent phase (Lundkvist et al. 1993).

The levels of total IgA as well as virus-specific IgA1 antibodies have been shown to be elevated during acute HFRS as well as during HCPS (Bostik et al. 2000, de Carvalho Nicacio et al. 2000, Padula et al. 2000). Furthermore, PUUV-specific IgA has still been detectable over 10 years after the infection (de Carvalho Nicacio et al. 2000). Moreover, total IgE as well as virus-specific IgE have been reported to be elevated during acute PUUV infection (Alexeyev et al. 1994).

The neutralizing antibodies develop early after hantavirus infection and they are usually present at the onset of the disease (Hörling et al. 1992). They control the

infection and in HCPS it has been shown that high neutralizing antibody titres in the acute phase associate with a milder clinical course of the disease (Bharadwaj et al. 2000). It is believed that hantaviruses leave a life-long immunity. In support of this assumption, PUUV IgG antibodies against N protein and Gn and Gc proteins as well as virus-neutralizing antibodies have been detected several decades after the infection (Settergren et al. 1991, Hörling et al. 1992, Lundkvist et al. 1993). The humoral cross-reactivity between different hantaviruses is high, particularly between viruses carried by the same rodent genus (Lundkvist et al. 1997, Maes et al. 2004). It is not known, whether a previous hantavirus infection protects from an infection by another hantavirus.

2.3.9 Mechanisms of hantavirus pathogenesis

The pathogenesis of hantavirus infections remains presently unclear. However, the capillary leakage syndrome is a central phenomenon and many signs and symptoms of as well HFRS as HCPS can be explained by this phenomenon. Host genetics influence both the susceptibility to hantavirus infections and the severity of the disease. Immunological factors rather than direct cytopathy probably play an important role in the pathogenesis and the development of increased vascular permeability. Although signs of apoptosis have been detected during PUUV infection, they have been attributed to immune responses. The most important immunological factors considering the pathogenesis of hantavirus infection are probably cytokines and T cells. An imbalance in the production of pro-inflammatory and anti-inflammatory cytokines, which could downregulate the early pro-inflammatory immune response, may be important. The virus-specific cytotoxic T cells are assumed to play an important role in the development of endothelial cell dysfunction and capillary leakage. However, also inefficient control of effector T cells by Tregs may contribute to the pathogenesis. The exact mechanisms behind the increased vascular permeability are unclear. However, the role of VEGF interacting with β_3-integrin may be important. Furthermore, the complement system may also be involved in the development of capillary leakage. The virus-specific neutralizing antibodies play a role in the control of infection and the evolvement of the long-lasting immunity.

2.4 Pentraxins

Pentraxins are a family of acute-phase pattern recognition receptor (PRR) proteins, which are characterized by a cyclic multimeric structure (Garlanda et al. 2005, Bottazzi et al. 2009). PRRs recognize microbial structures called pathogen-associated molecular patterns and activate the innate immune response (Garlanda et al. 2005). Members of the pentraxin family include the short pentraxins, i.e. CRP and serum amyloid P, as well as the long pentraxins, e.g. PTX3 and neuronal pentraxin (Garlanda et al. 2005).

2.4.1 C-reactive protein

CRP was originally described and named for its ability to bind in a calcium-dependent way the C-polysaccharide of *Streptococcus pneumoniae* (Tillett and Francis 1930, Abernethy and Avery 1941). CRP is the prototype of an acute phase response protein produced in the liver in various inflammatory and infectious conditions mainly in response to IL-6 (Volanakis 2001, Garlanda et al. 2005). The main functions of CRP are the activation of the classical complement pathway, enhancement of phagocytosis, and induction of cytokine production (Volanakis 2001, Ablij and Meinders 2002). CRP plays an important role in the innate immune response against different micro-organisms. It is rapidly increased up to 1,000-fold after the onset of a stimulus (Ablij and Meinders 2002). Numerous other conditions besides infections and inflammation, such as trauma, surgery, burns, necrosis, myocardial infarction, malignancies, childbirth, strenuous exercise, and stress, can stimulate the production of CRP (Volanakis 2001, Ablij and Meinders 2002). CRP may also have a role in the development of atherosclerosis and cardiovascular diseases (Ablij and Meinders 2002).

CRP is widely used in clinical practice in the evaluation of disease severity in different infections as well as inflammatory disorders. It is commonly used in assessing the severity and prognosis of various bacterial infections. However, in pneumonia as well as in sepsis, the results have been controversial concerning the predictive value of CRP in these conditions (Suprin et al. 2000, Pettilä et al. 2002, Muller et al. 2007, Chalmers et al. 2008, Silvestre et al. 2009). Furthermore, the

ability of CRP to discriminate or predict the outcome of sepsis has been disputed (Mitaka 2005, Silvestre et al. 2009, Tsalik et al. 2011).

CRP is also used in the context of distinguishing viral from bacterial infections, although its discriminative value has been debated (Salonen and Vaheri 1981, Gendrel et al. 1999, Heiskanen-Kosma and Korppi 2000). Although CRP concentrations tend to be higher in invasive bacterial infections than in viral infections, the concentrations overlap and concentrations of 100 mg/l or higher have been reported in different viral infections, including NE (Salonen and Vaheri 1981, Settergren et al. 1989, Mustonen et al. 1994a).

Studies concerning CRP and the prognosis of the viral infection are not very abundant. In children, CRP concentrations do not relate to the severity of influenza or adenovirus infection (Appenzeller et al. 2002, Edelbauer et al. 2006). In influenza A H1N1pdm09 virus infection, CRP has been shown to be an independent prognostic factor for intensive care unit admission and mechanical ventilation (Zimmerman et al. 2010). Furthermore, persistence of elevated CRP levels after treatment has related to poor prognosis in influenza A H1N1 (Wen et al. 2011). In severe acute respiratory syndrome (SARS), elevated CRP level at admission has been shown to be a predictive factor for death (Wang et al. 2004). In HIV, CRP is significantly related to all-cause mortality as well as disease progression (Neaton et al. 2010). This association is seen also in children (Drain et al. 2007). In acute hepatitis A virus infection, elevated CRP is an independent risk factor for the development of acute kidney injury (Choi et al. 2011). In dengue virus infection, elevated CRP levels associated with the severity of dengue hemorrhagic fever (Levy et al. 2010). In children with dengue virus infection, CRP concentrations were higher in dengue hemorrhagic fever patients than in patients with dengue fever (Juffrie et al. 2001). However, in Vietnamese children with dengue hemorrhagic fever, no association of CRP with disease severity could be detected (Bethell et al. 1998). In Crimean-Congo hemorrhagic fever, CRP proved to be a risk factor for severe clinical course of the disease and fatality (Yilmaz et al. 2010, Ozturk et al. 2012).

In acute renal failure (ARF), there are reports of elevated CRP levels associating with poor disease outcome and death, but also reports with no significant difference in CRP levels between survivors and non-survivors have been published (Simmons et al. 2004, Wang et al. 2006, Kadiroglu et al. 2007, Perez Valdivieso et al. 2008,

Xie et al. 2011). However, contrary to the findings in ARF, it has been shown in a mouse model of systemic lupus erythematosus (SLE) that treatment with CRP prolongs survival and prevents and ameliorates proteinuria and nephritis (Du Clos et al. 1994, Rodriguez et al. 2005, Rodriguez et al. 2006). Transgenic SLE-prone mice expressing human CRP also have less proteinuria and a better life expectancy than the non-transgenic SLE-prone mice (Szalai et al. 2003). These findings have been attributed to the ability of CRP to increase the clearance of immune complexes and to prevent their accumulation in the renal cortex (Szalai et al. 2003). In humans, it has also been reported that certain genetic factors that associate with reduced CRP production predispose to the development of SLE (Russell et al. 2004).

In conclusion, CRP is the prototype acute phase protein produced in infection and inflammation, but also in various other clinical conditions. It plays an important role in the host innate immune responses against different micro-organisms. CRP is widely used in clinical practice in order to detect an infection, distinguish bacterial from viral infections, and to assess the severity and prognosis of different infections as well as inflammatory disorders.

2.4.2 Pentraxin-3

PTX3 is a multifunctional PRR and the prototype protein of the long pentraxin group. In contrast to CRP, which is synthesized mainly in the liver, PTX3 is produced at the site of inflammation mainly by mononuclear phagocytes and dendritic cells, but also other types of cells, such as endothelial cells and fibroblasts (Mantovani et al. 2008, Bottazzi et al. 2009, Deban et al. 2009). Also renal epithelial cells produce PTX3 in response to stimulation (Nauta et al. 2005). Neutrophil granules can serve as a source of pre-synthesized PTX3, which upon stimulation, is released rapidly into circulation (Jaillon et al. 2007). The production of PTX3 is induced by pro-inflammatory signals, such as by bacterial lipopolysaccharide, IL-1β, TNF-α, and TLR engagement, but not by IL-6 (Mantovani et al. 2008, Bottazzi et al. 2009, Deban et al. 2009). PTX3 has the capacity to bind complement component C1q and to activate the classical complement pathway (Bottazzi et al. 1997). It also interacts with factor H, an alternative pathway regulator thus preventing exaggerated complement activation (Deban et al. 2008). It interacts with

several bacteria, fungi, and viruses, promoting their phagocytosis and clearance (Mantovani et al. 2008, Bottazzi et al. 2009, Deban et al. 2009). In addition, PTX3 has a non-redundant role in female fertility by acting as a nodal point for the assembly of the cumulus oophorus hyaluronan-rich extracellular matrix necessary for successful ovulation (Garlanda et al. 2005).

PTX3 has been studied in various conditions. PTX3 concentration has been found to associate with cardiovascular risk factors and act as an early indicator of acute myocardial infarction (Peri et al. 2000, Jylhävä et al. 2011a). It predicts mortality in coronary heart disease and myocardial infarction as well as after ischemic stroke (Latini et al. 2004, Dubin et al. 2012, Ryu et al. 2012). In vasculitis, it indicates disease activity (Fazzini et al. 2001, Dagna et al. 2011). In addition, patients with a chronic kidney disease show an increase in PTX plasma levels, with the highest levels in patients with the most severe form of the disease (Tong et al. 2007).

PTX3 has an essential role in anti-fungal innate immune response. PTX3-deficient mice are highly susceptible to *Aspergillus fumigatus* infection (Garlanda et al. 2002). Furthermore, the administration of PTX3 has protective efficacy against *Aspergillus* challenge in mice with bone marrow transplants and potentiates the protective effect of amphotericine B (Garlanda et al. 2002, Gaziano et al. 2004).

In the context of bacterial infections, previous studies have shown that high PTX3 levels are associated with higher mortality in septicemia and septic shock, as well as indicate shock in severe meningococcal disease (Sprong et al. 2009, Mauri et al. 2010, Huttunen et al. 2011a). In addition, high PTX3 is an early predictor of bacteremia and septic shock in hematologic patients with neutropenic fever (Vänskä et al. 2011). In critically ill patients, PTX3 correlates with disease severity and infection and furthermore, in febrile patients at the emergency department, it predicts severe disease (Muller et al. 2001, de Kruif et al. 2010). In addition, in leptospirosis, elevated PTX3 levels predict disease severity and mortality (Wagenaar et al. 2009). Increased PTX3 levels also associate with active *Mycobacterium tuberculosis* infection (Azzurri et al. 2005).

In viral infections, previous clinical studies concerning PTX3 are rare. PTX3 has been studied in patients with dengue virus infection and the concentrations were detected to be higher in patients suffering from dengue shock syndrome than in patients with dengue fever or dengue hemorrhagic fever (Mairuhu et al. 2005). *In*

vitro and in mouse models, PTX3 has been found to have antiviral activity against influenza virus (Reading et al. 2008) and also to protect from murine and human cytomegalovirus (CMV) infection (Bozza et al. 2006).

To conclude, PTX3 plays an important role in innate immunity, including complement activation, opsonization and regulation of inflammation. Pathogen recognition and elimination are the main functions of PTX3. Moreover, clinical observations point to PTX3 as a rapidly responding marker of infection and inflammation, as well as tissue damage.

2.5 Interleukin-6

The IL-6 molecule is a multifunctional cytokine involved in immune responses and inflammation and also in the regulation of metabolic, regenerative, and neural processes (Papanicolaou et al. 1998, Scheller et al. 2011). It is mostly regarded as a pro-inflammatory cytokine, but IL-6 has also many anti-inflammatory activities. It plays a pivotal role during the transition from innate to acquired immunity (Scheller et al. 2011). IL-6 is necessary for T cell recruitment and also acts in anti-apoptosis and differentiation of T cells. Further, it plays a crucial role in B cell proliferation and differentiation as well as in the production of immunoglobulins. IL-6 also activates the production of acute-phase proteins. It is produced by various types of cells, such as monocytes, macrophages, lymphocytes, fibroblasts, and endothelial cells (Papanicolaou et al. 1998). IL-6 stimulates the cells via a specific IL-6 receptor.

Plasma IL-6 has been shown to be elevated in several clinical conditions, such as acute lung injury, acute myocardial infarction, and congestive heart failure and it was shown to predict morbidity and mortality in these conditions (Parsons et al. 2005, Geppert et al. 2006, Marcucci et al. 2006). Several studies have also shown plasma IL-6 levels to be elevated in ARF (Himmelfarb et al. 2004, Simmons et al. 2004, Åhlström et al. 2004, Gueret et al. 2009). In addition, high IL-6 levels predict higher mortality in ARF (Simmons et al. 2004, Kadiroglu et al. 2007). Increased plasma IL-6 in patients with a critical illness may be due to multiple factors. Increased IL-6 production by stimulated macrophages in injured organs has been described (Kielar et al. 2005). ARF may also reduce serum cytokine clearance.

In the context of infectious diseases, plasma IL-6 has predicted septic shock, organ dysfunction, and mortality in septic patients (Hack et al. 1989, Oberholzer et al. 2005, Bozza et al. 2007). Several studies concerning IL-6 in pneumonia have been carried out and showed high IL-6 levels to predict mortality in this patient group (Christ-Crain and Opal 2010). In patients with an orthopedic joint prosthesis, elevated serum IL-6 has predicted periprosthetic infection (Di Cesare et al. 2005). In active pulmonary tuberculosis, IL-6 level is increased, but during therapy the levels decrease (Djoba Siawaya et al. 2009). Further, in leptospirosis, high levels of IL-6 were associated with increased mortality (Wagenaar et al. 2009). In invasive aspergillosis, IL-6 levels are elevated, and persistently high IL-6 levels predict poorer disease outcome after initiation of treatment (Chai et al. 2010).

IL-6 levels have been detected to be elevated and to predict disease severity and mortality in several viral infections. However, this is not the case in all viral infections. In influenza, both plasma and nasal fluid levels of IL-6 have been associated with higher disease severity (Hayden et al. 1998, Kaiser et al. 2001). In HIV, IL-6 is strongly related to all-cause mortality (Kuller et al. 2008). In Chikungunya virus infection, IL-6 levels are elevated, they associate with a more severe disease, and remain elevated in chronic cases (Ng et al. 2009, Chopra et al. 2012). Studies on IL-6 in patients with respiratory syncytial virus (RSV), hepatitis B (HBV), or HCV infection have produced controversial results. Elevated levels have associated with both a less and a more severe clinical course of RSV infection (Bennett et al. 2007, Elliott et al. 2007). In children, IL-6 levels did not reflect the inflammatory activity of HBV infection, while in adults, undetectable IL-6 level at the early stage of acute exacerbation of HBV infection correlated with a more favorable long-term outcome (Gora-Gebka et al. 2003, Pan et al. 2011). In HCV infection, high IL-6 levels have predicted virological response to therapy (Kishida et al. 2009). However, in HIV/HCV coinfected patients, high plasma IL-6 levels have predicted failure of the treatment (Guzman-Fulgencio et al. 2012). In tick-borne encephalitis, the serum IL-6 levels did not correlate with the clinical severity of the disease, whereas in Japanese encephalitis, the cerebrospinal fluid levels of IL-6 were higher in non-survivors as compared to survivors (Winter et al. 2004, Toporkova et al. 2008).

In the context of hemorrhagic fevers, studies on IL-6 have produced controversial results. In dengue virus infection, high IL-6 levels have predicted severe disease,

dengue hemorrhagic fever and mortality (Juffrie et al. 2001, Chen et al. 2006, Bozza et al. 2008). However, a Costa Rican study or another one conducted among Vietnamese children did not find any significant association of IL-6 levels with the severity of dengue virus infection (Bethell et al. 1998, Avila-Aguero et al. 2004). In ebola virus infection, elevated IL-6 levels have predicted both fatal and non-fatal infection (Baize et al. 2002, Wauquier et al. 2010). These conflicting results may be due to several factors, such as differences in the timing of sample collection and classification of the disease severity. A strong correlation between survival and low IL-6 level has been demonstrated in Lassa fever, while in Crimean-Kongo hemorrhagic fever, IL-6 level did not differ between severe and mild cases (Papa et al. 2006, Branco et al. 2011). In hemorrhagic fever-like illness caused by a novel Bunyavirus, the Huaiyangshan virus, higher IL-6 levels were associated with fatal outcome (Zhang et al. 2012). Studies concerning IL-6 in hantavirus infections have been reviewed earlier in this thesis.

To add up, IL-6 is a multifunctional cytokine and one of the most important pro-inflammatory cytokines responsible for the production of acute phase reactants, fever and septic shock together with IL-1 and TNF-α. In viral infections, high IL-6 levels have been related to both more and less severe clinical diseases. This may be due to several factors, but differences in the timing of sample collection and assigning disease severity may play a role in these controversial results.

2.6 Indoleamine 2,3-dioxygenase enzyme

IDO is an enzyme catalyzing the first and rate-limiting step in the major pathway of degradation of the essential amino acid tryptophan to kynurenine and its derivatives (Mellor and Munn 2004, Mellor 2005). Thereby, IDO limits the availability of tryptophan. IDO is expressed widely in various immune cells, including antigen-presenting cells, such as monocyte-derived macrophages and dendritic cells (Mellor and Munn 2004, Mellor 2005). Also other types of cells express IDO, such as fibroblasts, tumor cells, and TECs (Mellor and Munn 2004, Mellor 2005, Mohib et al. 2007). Th1 type cytokine IFN-γ is the strongest inducer of IDO, however also other cytokines and bacterial lipopolysaccharide are capable of inducing it (Mellor and Munn 2004). Increased IDO activity results in the induction of Tregs and, by a

positive feedback loop, this can further enhance IDO activity by increased IFN-γ expression (Mellor and Munn 2004, Mulley and Nikolic-Paterson 2008). By reducing tryptophan, IDO inhibits the replication of various bacteria, intracellular parasites, and viruses, thus acting as an antimicrobial effector molecule (Mellor and Munn 2004).

Depletion of tryptophan can also lead to inhibition of T cell responses and proliferation and thus to immunosuppression and tolerance (Hwu et al. 2000, Mellor et al. 2002, Mellor and Munn 2004, Mellor 2005). In aged individuals, IDO activity is increased and predicts mortality, which may be the consequence of suppressed T cell function (Pertovaara et al. 2006). Placental IDO protects the fetus from rejection during pregnancy (Munn et al. 1998). IDO may also act as a protective negative regulator in some autoimmune disorders (Sakurai et al. 2002, Hayashi et al. 2004). Furthermore, it contributes to the escape of tumor cells from immune surveillance (Mellor et al. 2002, Uyttenhove et al. 2003).

Enhanced tryptophan degradation or increased IDO activity has been noted in several conditions, such as infections, autoimmune diseases, malignancies, neurological disorders, cardiovascular diseases, and depression (Schrocksnadel et al. 2006). In rheumatoid arthritis as well as in primary Sjögren's syndrome, the overexpression of IDO associates with disease severity and, in SLE, high IDO activity predicts disease activation (Pertovaara et al. 2005, Schroecksnadel et al. 2006, Pertovaara et al. 2007). IDO activity also associates with disease progression in several malignancies and Alzheimer's disease, as well as with the risk factors for cardiovascular diseases (Widner et al. 2000, Huang et al. 2002, Niinisalo et al. 2008, Suzuki et al. 2010).

In the context of bacterial infections, high IDO levels have recently been documented to associate with severe disease and mortality in sepsis and septic shock (Huttunen et al. 2010, Tattevin et al. 2010). Also, treatment with granulocyte-macrophage colony-stimulating factor resulted in reduced IDO levels in severe sepsis, possibly due to improved antibacterial defense (Schefold et al. 2010). Previous studies also implicate that enhanced tryptophan degradation may be associated with poor outcome and the development of sepsis in trauma patients (Pellegrin et al. 2005, Logters et al. 2009, Ploder et al. 2009). Moreover, IDO activity predicts poor prognosis in community acquired pneumonia and pulmonary tuberculosis (Suzuki et al. 2011, Suzuki et al. 2012).

In the case of viral infections, increased IDO activity has been found in acute dengue virus infection and chronic HCV infection (Larrea et al. 2007, Becerra et al. 2009). Increased tryptophan degradation has also been found in patients with chronic Epstein-Barr virus (EBV) and HIV infections (Fuchs et al. 1990, Bellmann-Weiler et al. 2008). In HIV patients, enhanced tryptophan degradation by IDO was also associated with disease progression and complications, such as weight loss and neuropsychiatric disorders (Fuchs et al. 1990, Schroecksnadel et al. 2007). Furthermore, antiretroviral therapy has reduced tryptophan degradation (Fuchs et al. 1990).

With regard to renal diseases, it has been documented that tryptophan metabolite levels increase in chronic renal insufficiency, presumably due to an increase in production and/or decrease in degradation rather than due to a decrease in renal excretion (Saito et al. 2000, Schefold et al. 2009). In kidney allograft recipients, increased IDO activity is associated with rejection (Brandacher et al. 2007). In mice, IDO promotes renal ischemia-reperfusion injury, while, in contrast, it acts as a protective factor in nephrotoxic serum nephritis, a model of crescentic glomerulonephritis (Mohib et al. 2008, Hou et al. 2009).

Taken together, IDO has multiple functions in the immune system. It acts as a part of the innate immunity in antimicrobial defense. On the other hand, IDO also has immunosuppressive functions. However, its precise role in different disease processes remains currently mostly unclear.

2.7 Cell-free DNA

Detectable amounts of cf-DNA are present in the plasma of healthy individuals (Zhong et al. 2007a). However, markedly elevated levels of cf-DNA have been detected in different clinical conditions and circulating nucleic acids have recently received growing attention. Furthermore, the discovery of fetus- and placenta-derived nucleic acids in maternal plasma has opened up a new possibility for prenatal monitoring of pregnancy-associated complications and fetal abnormalities (Lo et al. 1997, Swarup and Rajeswari 2007).

High levels of cf-DNA have been reported in various conditions, such as in cancer, autoimmune diseases, myocardial infarction, stroke, pre-eclampsia, organ

transplant rejection, and trauma (Lo et al. 1999, Lo et al. 2000, Jahr et al. 2001, Rainer et al. 2003, Antonatos et al. 2006, Gadi et al. 2006, Zhong et al. 2007b, Mosca et al. 2009). Cf-DNA has also been proposed as a marker for outcome or disease severity in these conditions (Butt and Swaminathan 2008). In critically ill patients, cf-DNA has also predicted in-hospital mortality (Saukkonen et al. 2007). In addition, a recent study showed that in aged women, cf-DNA levels are elevated (Jylhävä et al. 2011b). The current view is that in different diseases, cf-DNA originates from apoptotic or necrotic cells and therefore reflects the amount of cellular damage (Jahr et al. 2001). This hypothesis is corroborated by the frequent observation of cf-DNA having a nucleosomal (150-200 base pairs (bp) in length) or ladder-like appearance characteristic of apoptotic cells (Jahr et al. 2001, Langford et al. 2007).

Concerning infectious diseases, studies in sepsis patients indicate that plasma cf-DNA levels are elevated in septic patients with poor outcome (Zeerleder et al. 2003, Rhodes et al. 2006, Saukkonen et al. 2008, Huttunen et al. 2011b). In trauma patients, cf-DNA has predicted inflammatory second hit and sepsis, while in febrile patients, it has showed prognostic value in assessing the probability and severity of infection and sepsis (Margraf et al. 2008, Moreira et al. 2010). In the context of viral infections, the studies concerning cf-DNA are few. Elevated levels of cf-DNA have been found in patients with HBV infection (Bhargava et al. 2010). In a recent study in dengue patients, the cf-DNA levels were elevated as well as they correlated with the severity of the infection (Ha et al. 2011).

Circulating DNA can cross the kidney barrier (Botezatu et al. 2000). However, the exact mechanism by which it crosses the glomerular basement membrane is currently unknown. Urinary cf-DNA has previously been studied in various conditions. Colorectal cancer patients secrete cf-DNA with K-ras mutations into urine (Su et al. 2004, Su et al. 2005). Also, fetal Y-chromosomal DNA sequences have been detected in the urine of pregnant women carrying male fetuses (Koide et al. 2005, Shekhtman et al. 2009). In hematopoetic stem cell transplant patients, donor-derived DNA has been detected in urine, whereas in the case of renal transplantation recipients, donor-derived DNA has been found in urine and also suggested to be a marker for acute transplant rejection (Zhang et al. 1999, Hung et al. 2009). With reference to infections, cf-DNA has been demonstrated to be

elevated in the urine of patients with urinary tract infection (Garcia Moreira et al. 2009). In viral infections, urinary cf-DNA has not previously been studied.

To conclude, plasma cf-DNA reflects the amount of cellular damage in infection, inflammation and tissue trauma and it has been suggested as a prognostic marker in these conditions. Plasma and urinary cf-DNA also present as possible non-invasive diagnostic tools in different conditions, such as in pregnancy, organ transplantation and cancer.

3. AIMS OF THE STUDY

The aims of this study were:

1. To study whether plasma CRP and IL-6 levels are associated with the severity of NE and whether their levels have prognostic significance (I).

2. To determine whether plasma PTX3 level is associated with disease severity in NE and to evaluate the possible role of PTX3 in the pathogenesis of NE (II).

3. To evaluate the association of serum IDO concentration with disease severity and the possible role of IDO in the pathogenesis of NE (III).

4. To assess plasma cf-DNA levels and urinary cf-DNA excretion in acute NE as well as their associations with the severity of the disease (IV).

4. SUBJECTS AND METHODS

4.1 Patients

The patients in all Studies I-IV were prospectively collected consecutive patients treated at the Tampere University Hospital, Finland, for NE. The diagnosis was serologically confirmed in all cases. The patients were collected as three groups of patients at different time periods (Table 2).

The patients in Study I (concerning CRP and IL-6) included patients from groups 1 and 2. Group 1 comprised 70 patients and group 2 comprised 61 patients. Thirteen patients in group 2 were excluded from Study I due to lack of IL-6 determinations. Finally, 118 patients treated between September 1997 and December 2004 constituted the study cohort in Study I.

The study cohort in Study III (dealing with IDO) consisted of group 2 with 61 patients and group 3 with 41 patients, altogether 102 patients treated between January 2000 and January 2008.

Group 2, with 61 patients treated between January 2000 and December 2004 comprised the study cohort in Studies II (PTX3-study) and IV (cf-DNA-study). Thus, 48 patients treated between January 2000 and December 2004 were included in all Studies I-IV. Furthermore, 172 patients altogether were studied in Studies I-IV. Table 2 shows the patient groups and study cohorts as well as the time periods when the patients of each group were hospital-treated for acute NE. The clinical characteristics of the patients in Studies I-IV are shown in Table 3.

Table 2. Number of patients in three different patient groups and in Studies I-IV. The time period, when the patients were collected, is also shown.

	Group 1 N=70	Group 2 N=61	Group 3 N=41	Total number of patients
Hospital treatment	9/1997-12/1999	1/2000-12/2004	1/2005-1/2008	
Study I	70 patients	48 patients		118
Study II		61 patients		61
Study III		61 patients	41 patients	102
Study IV		61 patients		61

Table 3. Clinical characteristics of the patients in Studies I-IV.

	Study I	Study III	Studies II & IV
Patients[a]	118	102	61
Male/female[a]	86/32	69/33	44/17
Age (years)[b]	40 (15-71)	46 (22-77)	46 (22-77)
BMI (kg/m^2)[b]	25.0 (17.1-41.9)	26.2 (18.5-37.0)	25.1 (19.8-35.7)

[a]number, [b]median (range)

4.2 Methods

4.2.1 Study protocols

The patients in group 1 were first recruited in the study by clinical suspicion of acute NE. Patients in group 2-3 were recruited in the study by clinical suspicion and a positive rapid point-of-care PUUV test (Hujakka et al. 2001). Thereafter, patients with a negative serological enzyme immunoassay test for PUUV infection (Vapalahti et al. 1996) were excluded from further analyses in all patient groups. In addition, patients with no informed consent were excluded from all patient groups. Group 3 also had other inclusion and exclusion criteria. The inclusion criteria were: age 16 years or older, history of fever for one week or less, and at least one of the following criteria: dipstick proteinuria $\geq$2+, serum creatinine >200 μmol/l, blood hematocrit $\geq$45 %, or platelet count $\leq$150 x10^9/l. The exclusion criteria were: pregnancy, positive bacterial culture from a sterile body site, clinical diagnosis of a bacterial infection as etiology of the febrile illness, and underlying, chronic renal disease.

All patients in Studies I-IV were studied during the acute phase of NE. A detailed past and current medical history was obtained, and a careful physical examination was performed. All blood specimens were obtained either on admission or after that between 7:30-9:30 in the morning. From patients in group 1, the first blood sample was obtained on the first morning after hospital admission, and from other patients, on the first weekday morning. Thereafter, blood samples were taken on consecutive mornings. In Study I, the blood samples were taken on three consecutive mornings and, in Studies II-IV, on up to five consecutive mornings.

In Study I, urine collection started on the first evening of hospital care and continued until three days were completed. The nightly collection period was set from the last voiding at bedtime until the last voiding on rising. The daytime collection commenced immediately thereafter and was maintained until a total of 24 hours were completed. After completion, the volumes were measured and timing was recorded for the collection periods.

In each Study I-IV, the highest and the lowest value of each patient of the various variables measured during hospitalization were designated as the maximum and minimum values. In Studies I-III, certain definitions were made. In Study I, high plasma creatinine, CRP and IL-6 were defined as a value exceeding the median maximum level among the study population (193 µmol/l, 69 mg/l and 14.05 pg/ml, respectively) and thrombocytopenia was defined as the minimum platelet count equal to or lower than the median among the study population (66 x 10^9/l). In Study II, clinically significant thrombocytopenia was defined as a minimum platelet count lower than 50 x 10^9/l. Finally, in Study III, significant renal insufficiency was defined as a plasma creatinine value exceeding 250 µmol/l and minimum urinary output was defined as low if it was equal to or lower than the median in the study population (1,440 ml/day).

The patients in group 2 were examined at the out-patient clinic 10 and 30 days after discharge from the hospital. The out-patient clinic controls to the patients in group 3 were performed 15 days, as well as six and 12 months after the fever ceased.

4.2.2 Puumala virus serology

A rapid immunogromatographic PUUV IgM test, Point-of-care PUUMALA® (Reagena, Toivala, Finland) was used at admission to hospital (Hujakka et al. 2001). However, the serological diagnosis was verified and based on an immunoglobulin M-capture enzyme immunoassay and PUUV Sotkamo strain full-length N protein expressed by the baculovirus system in Sf9 insect cells (Vapalahti et al. 1996). In comparison with various other protocols, the assay has showed optimal sensitivity and specificity (Sjölander et al. 1997).

4.2.3 C-reactive protein, pentraxin-3 and interleukin-6 determinations

Plasma CRP was analyzed by Hitachi 705 E Analyzer from 1997 to 1998 and, after that, by the Roche Diagnostics CRP method using Cobas Integra analyzer (F.Hoffman-La Roche Ltd, Basel, Switzerland).

Plasma PTX3 concentrations were determined afterwards from frozen samples stored at −70°C. The determinations were performed by using a commercially available human PTX3 immunoassay (Quantikine, R&D Systems, Inc., Minneapolis, MN), following the manufacturer's instructions.

Plasma IL-6 concentrations were determined afterwards from frozen samples stored at –70 °C by using commercially available enzyme-linked immunosorbent assays (PeliKine Compact human IL-6 kits; Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), following the manufacturer's instructions. The detection limit for the assay was 0.4 pg/ml for IL-6. None of the 118 patients had values below the detection limit.

4.2.4 Indoleamine 2,3-dioxygenase determinations

Serum tryptophan and kynurenine concentrations were measured afterwards from frozen samples stored at –70 °C by reverse-phase high-performance liquid chromatography (HPLC), as previously described (Laich et al. 2002). Tryptophan was separated with a Shimadzu liquid chromatograph LC-10AD VP (Shimadzu Co, Kyoto, Japan) using a 50-mm BDS Hypersil C18 5 μm column (Thermo Electron Co, Bellefonte, PA). It was monitored by fluorescence with a Shimadzu RF-10A XL detector at 266 nm excitation and 366 nm emission wavelengths. Kynurenine was separated with a Hewlett Packard 1100 liquid chromatograph (Palo Alto, CA) using a Merck LiChroCart 55-4,150 mm cartridge containing a Purospher STAR RP-18 3 μm column (Merck Co, Darmstadt, Germany). It was determined by ultraviolet absorption at 360-nm wavelength with a Hewlett Packard G13144 detector. The rate of tryptophan degradation reflects the IDO enzyme activity, and IDO level can thus be measured by determining the ratio of kynurenine to tryptophan (kynurenine/tryptophan) (Schrocksnadel et al. 2006). The kynurenine/tryptophan ratio was calculated by relating concentrations of kynurenine to tryptophan, this allowing estimation of IDO activity.

4.2.5 Cell-free DNA

4.2.5.1 Quantification analyses of cell-free DNA

The cf-DNA analyses were performed afterwards from frozen samples stored at -70 ºC. The amount of total cf-DNA was determined directly in plasma and urine without any DNA purification step, using the Quant-iT™ high-sensitivity DNA assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Plasma samples were analyzed in duplicate and the mean of the two values was used as the final value. The assessed intra-day variation coefficients at the mean plasma cf-DNA levels of 0.673 µg/ml, 0.876 µg/ml, and 1.59 µg/ml were 4.2 %, 1.0 %, and 4.1 %, respectively. The corresponding inter-day variation coefficients were 5.5 %, 4.3 %, and 6.6 %. Total cf-DNA in urine was measured in 20/61 patients with quadruple measurements in which the mean of the four values was used as the final value. The assessed intra-day variation coefficients at the mean urine levels of 0.307 µg/ml, 0.769 µg/ml, and 1.13 µg/ml were 5.0 %, 4.8 %, and 3.5 %, respectively. The corresponding inter-day variation coefficients were 10.0 %, 6.5 %, and 10.2 %. Timed overnight urinary excretion of cf-DNA was calculated as follows: (concentration x total volume)/(time span).

4.2.5.2 Extraction and qualitative analyses of cell-free DNA

Qualitative analysis of plasma and urine cf-DNA was performed for randomly selected 10 patients with and without renal insufficiency (defined as maximum plasma creatinine >370 µmol/l and maximum plasma creatinine <125 µmol/l, respectively). Plasma and urine cf-DNA was extracted using the NucleoSpin® Plasma XS Kit (MACHEREY-NAGEL GmbH & Co., Düren, Germany), designed for isolation of low-molecular-weight (50-1000 bp) cf-DNA. Cf-DNA isolation was performed according to the manufacturer's instructions following the high-sensitivity protocol. Extracted cf-DNA samples were analyzed with the High Sensitivity DNA assay kit and an Agilent 2100 Bioanalyzer equipped with Expert 2100 software according to the manufacturer's instructions (Agilent Technologies Inc., Santa Clara, CA). Agilent 2100 Bioanalyzer uses a lab-on-a-chip technology to perform gel electrophoresis; nucleic acids are separated analogously to a capillary

electrophoresis and normalized to a ladder and two DNA markers, after which the software automatically calculates the size of each band. For each plasma sample, the appearance and intensity of low-molecular weight cf-DNA was estimated visually and graded as follows: 1=no visible cf-DNA or extremely weak band intensity, 2=intermediate band intensity, 3=strong band intensity. The researcher responsible for analyzing and grading the cf-DNA samples was blinded to the clinical data of the patients. The appearance of cf-DNA in urine was analyzed descriptively.

4.2.6 Complement analyses

The complement analyses were performed at the Department of Bacteriology and Immunology of the Haartman Institute and at the Helsinki University Central Hospital Laboratory. Plasma SC5b-9 measurements were performed using a commercial ELISA kit (Quidel, San Diego, CA) and plasma C3 concentrations were measured by nephelometry (Dade Behring, Marburg, Germany). Some samples did not meet the quality control criteria for the measurements of C3 and SC5b-9 levels defined by the manufacturer. Thus, these samples were excluded from the respective analyses.

4.2.7 Analytical methods

Blood cell count was completed by hematological cell counters by Bayer. From 1997 to June 1999, creatinine was determined in serum by Vitros (Johnson & Johnson, Rochester, NY, USA) and, after that, in plasma by Cobas Integra analyzer. Creatinine concentrations showed 10 % lower values after June 1999 than during the earlier years due to the change in the determination method. Therefore, in Study I the results of serum creatinine concentrations from September 1997 to June 1999 were multiplied by a coefficient of 0.9. Plasma sodium levels were analyzed using a Cobas Integra analyzer (F.Hoffman-La Roche Ltd., Basel, Switzerland). The 24-hour urinary protein excretion was measured by the pyrogallolal red molybdate method (Olli C.; Kone Instruments, Helsinki, Finland) from 1997 to April 1998 and, after that, by Cobas Integra analyzer.

4.2.8 Chest X-ray findings

If a chest radiograph revealed heart enlargement, an increase in venous stasis, interstititial or alveolar edema, a lung infiltration, atelectasis or pleural effusion, it was defined as pathologic (Paakkala et al. 2004b).

4.2.9 Statistical analyses

In order to describe the data, medians and ranges are given for continuous variables, and numbers and percentages for categorical variables.

In Study I, the patients were divided into two groups in order to evaluate the associations of plasma IL-6 and CRP values with the severity of NE, first according to the maximum IL-6 value and then according to the maximum CRP value. For the purpose of further evaluation of the patients, they were divided into four groups using median values of IL-6 and CRP as cut off points: group 1 with low IL-6 and low CRP (equivalent to or lower than the median), group 2 with low IL-6 and high CRP (higher than the median), group 3 with high IL-6 and low CRP, and group 4 with both high IL-6 and high CRP. In Studies II and III, the patients were divided into two groups according to the maximum PTX3 and IDO values to evaluate the associations of plasma PTX3 and IDO levels, respectively, with the severity of NE. The cut off points for maximum PTX3 and IDO values were determined with receiver operating characteristics (ROC) analyses (Boyd 1997).

Groups were compared using the Mann-Whitney *U*-test or Kruskal-Wallis test, as appropriate. Categorical data were analyzed by the x^2 test or the Fisher's exact test. Correlations were calculated by means of Spearman's rank correlation coefficient. Wilcoxon's test was used to compare two related samples.

Logistic regression analyses were also performed in Studies I and III. In Study I, the analyses were performed with high serum creatinine, thrombocytopenia, or hospitalization exceeding seven days (median in the study population) as dependent factors and high plasma IL-6 and high plasma CRP as independent factors for the purpose of further examination of the associations of these factors with the severity of the disease. Age was also included in these models. In Study III, a logistic regression analysis with significant renal insufficiency (creatinine >250 μmol/l) as a dependent factor and high IDO and low urinary output as independent factors was

performed as to further examine the associations of these factors with significant renal insufficiency. Age and sex were also included in this model. Adjusted odds ratios (OR) and their 95 % confidence intervals (95 % CI) are given.

All tests were two-sided, and statistically significant *P*-values are given. All analyses were made with the SPSS (version 7.5 and 18) statistical software package.

4.2.10 Ethical considerations

All patients gave a written informed consent before participation in the study. All study protocols were approved by the Ethics Committee of Tampere University Hospital. The study protocol of Study II was also approved by the University of Massachusetts Medical School.

5. RESULTS

5.1 Characteristics of the study material (Studies I-IV)

5.1.1 Clinical data

The clinical findings in patients participating in Studies I-IV are presented in Table 4. The clinical picture did not differ between the studies, except for the occurrence of shock in three patients in Study I. In the other studies shock was not observed in any of the patients. In addition, chest radiographs revealed pathologic findings in 30 patients (32 %, n=96) in Study I, 33 patients (41 %, n=81) in Study III, and 13 patients (30 %, n=44) in Studies II and IV. No secondary bacterial infections were detected and all blood cultures were negative. All patients recovered.

Table 4. Clinical findings in patients in Studies I-IV.

	Study I (n=118)	Study III (n=102)	Studies II&IV (n=61)
Patients in shock[a]	3 (3 %)	0 (0 %)	0 (0 %)
Patients requiring dialysis[a]	6 (5 %)	5 (5 %)	4 (7 %)
Duration of fever before hospital admission (days)[b]	4 (1-14)	4 (1-15)	4 (1-15)
Hospitalization (days)[b]	7 (2-15)	6 (2-15)	6 (2-15)
Duration of fever (days)[b]	5 (2-15)	6 (2-19)	6 (2-19)
Change in body weight (kg)[b]	2.6 (0-12.0)	2.1 (0-12.0)	2.7 (0-12.0)
Urinary output min (ml/day)[b]	1,520 (50-7,000)	1,440 (50-4,940)	1,600 (50-4,940)
SBP min (mmHg)[b]	112 (82-162)	113 (74-170)	112 (82-162)

[a]number (percent), [b]median (range).
Abbreviations: min=minimum, SBP=systolic blood pressure

5.1.2 Laboratory variables

The laboratory findings in patients participating in Studies I-IV are presented in Table 5. The laboratory findings did not differ significantly between the groups. In addition, 24-hour urinary protein excretion was measured from 72 patients in Study I. The median maximum excretion was 1.80 g/day (range 0.14-17.78 g/day).

Table 5. Laboratory findings in patients in Studies I-IV.

	Study I (n=118)	Study III (n=102)	Studies II&IV (n=61)
Creatinine max (µmol/l)	193 (65-1,285)	176 (52-1,285)	175 (65-1,285)
Leukocytes max ($x10^9$/l)	10.0 (3.9-31.2)	10.1 (3.9-31.2)	9.9 (3.9-31.2)
Platelets min ($x10^9$/l)	66 (3-238)	61 (9-238)	68 (9-238)
Hematocrit min	0.36 (0.25-0.45)	0.36 (0.25-0.44)	0.36 (0.25-0.43)
CRP max (mg/l)	69 (11-269)	80 (16-269)	69 (17-269)

The values are presented as median (range).
Abbreviations: max=maximum, min=minimum, CRP=C-reactive protein

5.2 Plasma C-reactive protein and interleukin-6 levels and the outcome of nephropathia epidemica (Study I)

5.2.1 C-reactive protein

The median maximum plasma CRP in Study I was 69 mg/l. The patients were divided into two groups according to the maximum CRP value. The median age was higher in patients with high CRP (CRP>69 mg/l) than in patients with low CRP

(≤69 mg/l) (46 years, range 25-71 *vs* 38 years, range 15-64, *P* <0.001). The proportion of males and females did not differ between these two groups. Forty-two (72 %) of the patients with high CRP levels were males compared to 44 (73 %) of the patients with low CRP (*P* = 0.911). In addition, BMI did not differ between the two groups (median 26.2 kg/m^2, range 18.9-41.9 *vs* median 24.8 kg/m^2, range 17.1-37.2, high *vs* low CRP, *P*=0.298). The clinical and laboratory values reflecting the severity of the disease did not differ between patients with high and low CRP with the exception of minimum urinary output (Table 6). Furthermore, the occurrence of a pathologic chest radiograph had no significant association with high CRP values. Eighteen patients (37 %) with high CRP had a pathologic chest radiograph compared with 12 patients (26 %) with low CRP (*P* = 0.265).

Table 6. The clinical and laboratory parameters in 118 patients with acute NE divided into two groups according to the maximum plasma CRP value.

	CRP≤69 mg/l	CRP>69 mg/l	P-value
Duration of hospital stay (days)	6 (2-15)	7 (3-14)	0.222
Change in body weight (kg)	2.4 (0-12.0)	2.7 (0-9.9)	0.564
Urinary output min (ml/day)	1,700 (50-7,000)	1,400 (50-4,940)	0.035
SBP min	112 (82-162)	112 (86-158)	0.381
Urinary protein excretion max (g/day)	2.24 (0.14-10.00)	1.78 (0.30-17.78)	0.474
Creatinine max (µmol/l)	241 (65-1,285)	141 (68-1,156)	0.193
Leukocytes max (x10^9/l)	9.5 (3.9-31.2)	10.4 (5.4-26.8)	0.303
Platelets min (x10^9/l)	70 (3-238)	62 (13-187)	0.133

The values are presented as median (range).
Abbreviations: CRP=C-reactive protein, SBP=systolic blood pressure, min=minimum, max=maximum

5.2.2 Interleukin-6

The median of the maximum plasma IL-6 levels was 14.05 pg/ml. The patients were divided into two groups according to their maximum IL-6 value. The median age did not differ between the patients with plasma IL-6>14.05 pg/ml (high IL-6) and the patients with plasma IL-6≤14.05 pg/ml (low IL-6) (41 years, range 15-65 *vs* 39 years, range 17-71, *P* = 0.741). The proportion of males and females did not differ between the groups either. Forty-four (75 %) of the patients with high IL-6 were males compared with 42 (71 %) of the patients with low IL-6 (*P* = 0.679). In addition, BMI did not differ between these two groups (24.1 kg/m^2, range 17.1-37.2 *vs* 26.2 kg/m^2, range 19.2-41.9, high *vs* low IL-6 groups, *P*=0.179). The maximum level of plasma IL-6 associated with most variables reflecting the severity of the disease (Table 7). The occurrence of a pathologic chest radiograph had no significant associations with high IL-6. Twenty patients (38 %) with high IL-6 had a pathologic chest radiograph as compared to 10 patients (24 %) with low IL-6 (*P* = 0.147).

Table 7. The clinical and laboratory parameters in 118 patients with acute NE divided into two groups according to the maximum plasma IL-6 value.

	IL-6≤14.05 pg/ml	IL-6>14.05 pg/ml	P-value
Duration of hospital stay (days)	6 (2-15)	8 (3-14)	<0.001
Change in body weight (kg)	2.0 (0-9.9)	3.2 (0-12.0)	0.008
Urinary output min (ml/day)	2,180 (200-7,000)	1,040 (50-4,900)	<0.001
SBP min	115 (82-162)	110 (85-158)	0.034
Urinary protein excretion max (g/day)	1.68 (0.14-5.59)	2.51 (0.30-17.78)	0.017
Creatinine max (µmol/l)	140 (65-917)	242 (70-1,285)	0.057
Leukocytes max (x10^9/l)	9.0 (3.9-31.2)	11.9 (5.1-26.8)	0.001
Platelets min (x10^9/l)	80 (3-238)	55 (9-187)	<0.001

The values are presented as median (range).
Abbreviations: IL-6=interleukin-6, SBP=systolic blood pressure, min=minimum, max=maximum

5.2.3 C-reactive protein and interleukin-6

The patients in Study I were also divided into four groups according to the maximum CRP and IL-6 values. Patients in group 1 had both low CRP and IL-6, group 2 consisted of patients with high CRP and low IL-6, group 3 consisted of patients with low CRP and high IL-6, and patients in group 4 had both high CRP and IL-6. It was discovered that significantly fewer patients in group 2 with high CRP and low IL-6 had high creatinine level (>193 µmol/l) compared to the patients in the other three groups (group 2 *vs* group 1, 23 % *vs* 49 %, P =0.048; group 2 *vs* group 3, 23 % *vs* 70 %, P = 0.002; group 2 *vs* group 4, 23 % *vs* 53 %, P= 0.024). Furthermore, none of the patients in group 2 required dialysis treatment.

Logistic regression analyses were then performed to evaluate the association of high CRP and IL-6 values with high serum creatinine levels (>193 µmol/l), thrombocytopenia ($\leq 66 \times 10^9$/l) or hospitalization exceeding seven days. Age was also included in these models. High plasma CRP was found to be a protective factor for renal function in this model (Table 8). High plasma CRP did not have a significant association with thrombocytopenia or hospitalization exceeding seven days (data not shown). High plasma IL-6, on the contrary, was found to be an independent risk factor for high serum creatinine (Table 8). High IL-6 was also revealed as an independent risk factor for thrombocytopenia and hospitalization exceeding seven days (OR 3.6, 95 % CI 1.6-8.0, P=0.002, and OR 4.5, 95 % CI 1.9-10.8, P<0.001, respectively).

Table 8. Multivariate analysis of risk factors for serum creatinine level >193 µmol/l among 118 hospitalized patients with NE.

	Creatinine≤193 µmol/l (n=60)	Creatinine>193 µmol/l (n=58)	OR	95 % CI
Age (years)	38	42	1.0	0.99-1.06
High CRP				
-No	26	34	1	Reference
-Yes	34	24	0.3	0.13-0.74
High IL-6				
-No	36	23	1	Reference
-Yes	24	35	3.2	1.41-7.35

Abbreviations: CRP=C-reactive protein, IL-6=interleukin-6, OR=odds ratio, CI=confidence interval

5.3 Pentraxin-3 and the severity of NE (Study II)

The acute phase maximum PTX3 values were significantly higher as compared to the control values seen after hospitalization (median 42.0 ng/ml, range 3.9–1251.4 ng/ml vs. 1.1 ng/ml, range 0.4–6.6 ng/ml, $P<0.001$). The control samples were taken median 41 days (range 18-83 days) after the onset of fever.

To evaluate the association of PTX3 level with the parameters reflecting the severity of NE, correlations between different parameters were first calculated. A strong inverse correlation between the maximum plasma PTX3 level and the minimum blood platelet count was found ($r=-0.567$, $P<0.001$). Inverse correlations were also found between maximum PTX3 level and minimum C3 as well as minimum hematocrit levels ($r=-0.365$, $P=0.006$, and $r=-0.282$, $P=0.028$, respectively). The maximum PTX3 level correlated positively with the maximum blood leukocyte count ($r=0.477$, $P<0.001$) and the maximum hematocrit level ($r=0.390$, $P=0.002$), as well as with the maximum plasma IL-6 ($r=0.643$, $P<0.001$), serum IDO ($r=0.287$, $P=0.025$), and plasma SC5b-9 ($r=0.454$, $P<0.001$) levels.

Furthermore, the change in patient weight and the length of hospital stay (r=0.315, *P*=0.013, and r=0.401, *P*=0.001, respectively) correlated positively with the maximum PTX3 level.

ROC curves were then used to examine, whether the maximum plasma PTX3 level could act as an indicator of significant thrombocytopenia (minimum blood platelet value $<50\times10^9$/l). A maximum PTX3 level >101.6 ng/ml showed a sensitivity of 71 % and a specificity of 89 % for detecting significant thrombocytopenia, with an area under curve (AUC) value of 0.78 (95 % confidence interval [CI] 0.63–0.94). This cut-off point was used to divide the patients into two groups. In the low PTX3 group, the patients had a PTX3 maximum level ≤101.6 ng/ml, and in the high PTX3 group, the PTX3 maximum level was >101.6 ng/ml.

When comparing high and low PTX3 groups, high PTX3 level was found to associate also with several other parameters reflecting the severity of the disease (Table 9). However, in both groups two patients needed dialysis treatment and the proportion of such patients was not significantly different between the two groups (13 % *vs* 14 % high *vs* low PTX3 groups, *P*=0.251). Age and BMI did not differ between these two groups (median 45 years, range 25-60 *vs* median 47 years, range 22-77, *P*=0.657 and 28 kg/m^2, range 20-30 *vs* median 24 kg/m^2, range 20-36, *P*=0.579, high *vs* low PTX3 groups, respectively). Also the proportion of males and females did not differ between the two groups (70 % male *vs* 80 % male, *P*=0.524).

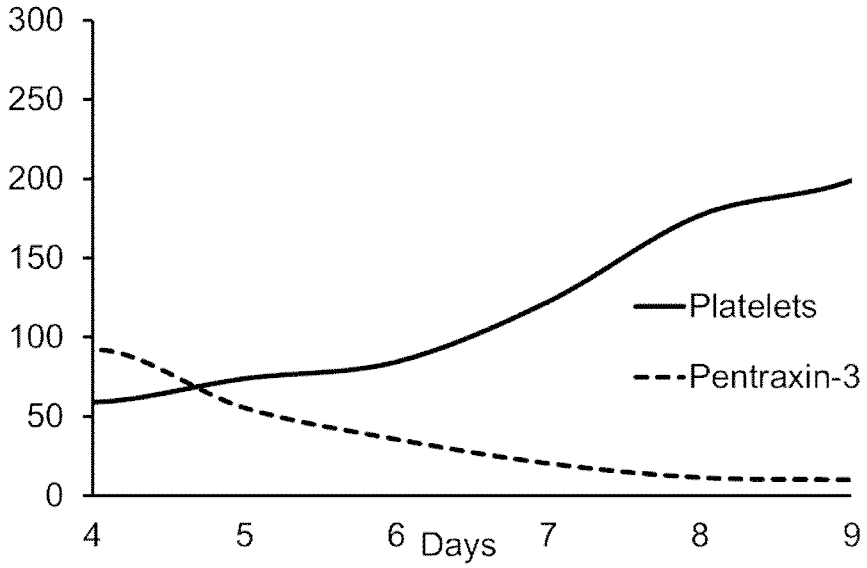
Table 9. The clinical and laboratory findings in 61 patients with NE divided into two groups according to the maximum PTX3 level.

	PTX3 max ≤101.6 ng/ml (n=46)	PTX3 max >101.6 ng/ml (n=15)	P-value
Hospital stay (days)	5 (2-15)	8 (4-14)	0.015
SBP min (mmHg)	110 (82-162)	112 (86-152)	0.585
Change in body weight (kg)	2.0 (0-10.1)	3.8 (0.5-12.0)	0.014
CRP max (mg/l)	68.4 (16.7-269.2)	75.3 (19.7-214.0)	0.331
IL-6 max (pg/ml) (n=48)	9.0 (1.3-44.8)	16.9 (6.8-96.6)	0.007
IDO max (µmol/mmol)	196.9 (46.6-1,044.7)	338.3 (119.2-3,679.2)	0.009
C3 min (g/l) (n=55)	1.29 (0.8-2.11)	1.07 (0.65-1.56)	0.017
SC5b-9 max (ng/ml) (n=57)	468.0 (103.5-903.5)	679.4 (238.4-1,034.0)	0.008
Platelets min (10E9/l)	77 (24-238)	36 (9-84)	<0.001
Leukocytes max (10E9/l)	9.7 (3.9-20.0)	16.1 (8.1-31.2)	<0.001
Hematocrit min	0.37 (0.30-0.43)	0.34 (0.25-0.38)	0.045
Hematocrit max	0.43 (0.35-0.57)	0.48 (0.34-0.54)	0.010
Creatinine max (µmol/l)	124 (65-1,156)	282 (113-1,285)	0.007
Sodium min (mmol/l)	132 (120-139)	129 (115-136)	0.028

Values are expressed as median (range).
Abbreviations: PTX=pentraxin-3, SBP=systolic blood pressure, CRP=C-reactive protein, IL-6=interleukin-6, IDO=indoleamine 2,3-dioxygenase, min=minimum, max=maximum

In order to examine the kinetics of the changes in the plasma PTX3 and blood platelet levels, their daily medians in relation to the day of the onset of fever were depicted. PTX3 level was at its highest (92.5 ng/ml) at the median time of hospital admittance, i.e., 4 days after the onset of fever, and, thereafter, it declined. The lowest platelet level (59×10^9/l), in turn, was also observed at the median time of admittance and the level rose thereafter (Figure 1).

Figure 1. Daily median pentraxin-3 (ng/ml) and blood platelet (x10^9/l) levels in relation to the onset of fever (day 0). (Adapted from Study II)



5.4 Indoleamine 2,3-dioxygenase and the degree of renal insufficiency (Study III)

The acute phase maximum IDO values were significantly higher than the convalescent phase values (median 199.3 µmol/mmol, range 46.6-3679.2 *vs* median 64.7 µmol/mmol, range 23.9-350.6, $P<0.001$). The control samples were taken median 22 (range 14-32) days after the onset of fever.

To evaluate the association of serum IDO level with the variables reflecting the severity of NE, correlations were calculated. A strong positive correlation was found between maximum serum IDO and creatinine levels as well as maximum IDO level and change in body weight (r=0.672, $P<0.001$, and r=0.526, $P<0.001$, respectively). Maximum IDO level and minimum urinary output were inversely correlated (r=-0.385, $P<0.001$). There was a positive correlation between maximum IDO level and the length of hospital stay as well as between maximum IDO and the maximum blood leukocyte count (r =0.494, $P<0.001$, and r=0.508, $P<0.001$, respectively).

The ability of maximum serum IDO level to function as an indicator of plasma creatinine level >250 µmol/l was evaluated using ROC curves. A maximum IDO>202 µmol/mmol showed a sensitivity of 85 % and a specificity of 75 % for detecting maximum plasma creatinine levels >250 µmol/l and the AUC was 0.84 (95 % CI 0.76–0.91). This cut-off point was then used to divide patients into two groups. Patients with a low IDO level had maximum IDO≤202 µmol/mmol and patients with a high IDO level had maximum IDO>202 µmol/mmol.

When comparing high and low IDO groups, a high IDO level was found to associate also with several other variables reflecting the severity of the disease (Table 10). Furthermore, all five patients who needed dialysis treatment were in the high IDO group ($P=0.025$). The patients in the high IDO group were older than the patients in the low IDO group (50 years, range 25-74 *vs* 38 years, range 22-77, $P=0.002$), while BMI did not differ between these two groups (27.1 kg/m^2, range 20.9-37.0 *vs* 24.8 kg/m^2, range 18.5-34.6, high *vs* low IDO groups, $P=0.149$). The

proportion of males and females did not differ between the two groups (70 % male *vs* 65 % male, high *vs* low IDO groups, *P*=0.618).

Table 10. The clinical and laboratory variables in 102 patients with NE divided into two groups according to the maximum IDO level.

	IDO max ≤202 µmol/mmol (n=52)	IDO max >202 µmol/mmol (n=50)	P-value
Hospital stay (days)	5 (2-15)	8 (3-14)	<0.001
Urinary output min (ml/day) (n=94)	1,900 (200-4,940)	1,100 (50-4,900)	<0.001
Change in body weight (kg)	1.0 (0-10.0)	3.5 (0-12.0)	<0.001
Creatinine max (µmol/l)	102 (52-537)	379 (75-1,285)	<0.001
CRP max (mg/l)	72.1 (15.9-176.0)	104.1 (19.7-269.2)	0.029
Leukocytes max (10^9/l)	9.0 (3.9-24.0)	11.9 (6.3-31.2)	<0.001
Hematocrit min	0.38 (0.31-0.44)	0.33 (0.25-0.40)	<0.001
Platelets min (10^9/l)	53 (14-172)	67 (9-238)	0.202

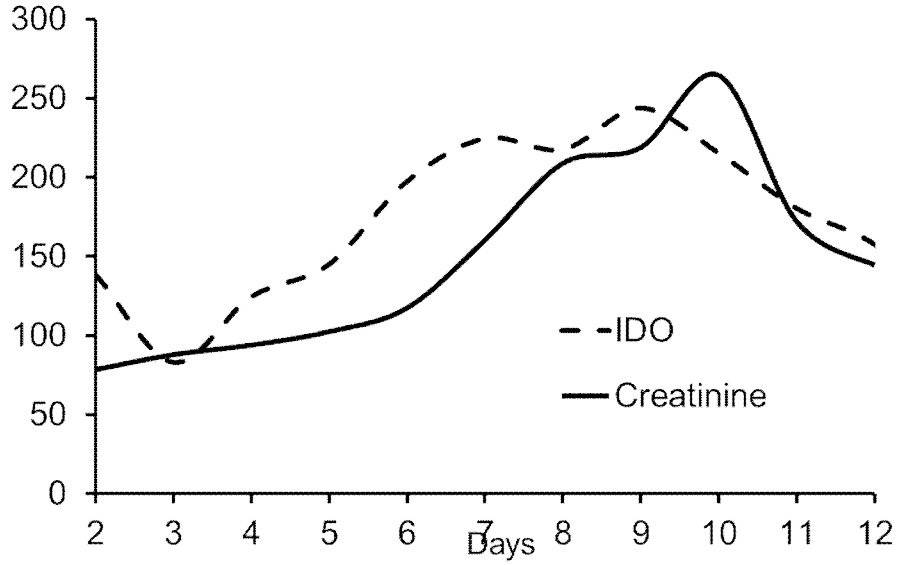
Values are expressed as median (range).
Abbreviations: IDO=indoleamine 2,3-dioxygenase, CRP=C-reactive protein, min=minimum, max=maximum

A logistic regression analysis was also performed in order to assess the association of high IDO level with significant renal insufficiency (plasma creatinine >250 µmol/l) when adjusted for age, sex and low urinary output (minimum urinary output ≤1,440 ml/day). Low urinary output was included in this model in order to detect the possible effect of decrease in renal excretion of kynurenine. High IDO level was revealed as an independent risk factor for significant renal insufficiency in this model (OR 17.57, 95 % CI 5.25-58.77, P<0.001). Age, sex or low urinary output did not have significant association with significant renal insufficiency in this analysis (data not shown).

Maximum kynurenine and tryptophan values were also analyzed separately by calculating correlations with the variables reflecting the severity of NE. Maximum kynurenine level was positively correlated with the maximum serum creatinine level and change in body weight (r=0.785, P<0.001, and r=0.517, P<0.001, respectively), as well as with the length of hospital stay and maximum blood leukocyte value (r=0.517, P<0.001 and r=0.516, P<0.001, respectively). It was inversely correlated with the minimum urinary output (r=-0.357, P<0.001). Maximum tryptophan level had only a weak positive correlation with the minimum urinary output (r=0.230, P=0.026), and a weak inverse correlation with the change in body weight, maximum blood leukocyte and maximum CRP values (r=-0.201, P=0.044; r=-0.244, P=0.014, and r=-0.256, P=0.009, respectively).

In order to analyze the kinetics of the changes in serum IDO and plasma creatinine levels, their daily medians in relation to the onset of fever were depicted. It was revealed that both variables first gradually increased to their peak values, after which they started to decline. Median IDO hit its peak value 243,9 µmol/mmol 9 days after the onset of fever and median creatinine was at its highest (265 µmol/l) one day later (Figure 2).

Figure 2. Daily median serum IDO (µmol/mmol) and creatinine (µmol/l) levels in relation to the onset of fever (day 0). (adapted from Study III)



5.5 Cell-free DNA in acute Puumala virus infection (Study IV)

The median of the maximum total plasma cf-DNA levels during the acute phase was 1.33 µg/ml, range 0.94-3.29. It was significantly higher than the median of the control values (0.77 µg/ml, range 0.55-0.99 µg/ml, $P<0.001$). The median maximum urinary excretion of cf-DNA during the hospitalization period was 0.68 µg/min (range 0.34-1.38). It was not increased as compared to the control values (median 0.62 µg/min, range 0.19-1.15, $P=0.43$). The control samples were taken median 41 (range 18-83) days after the onset of fever.

Correlations were calculated to evaluate the association of plasma cf-DNA with the variables reflecting the severity of NE. The maximum plasma cf-DNA levels correlated positively with the maximum blood leukocyte count, maximum plasma PTX3 levels, and the length of the hospital treatment (Table 11). There was also an inverse correlation between the maximum plasma cf-DNA levels and minimum

blood platelet count (Table 11). The maximum plasma cf-DNA levels did not correlate with the maximum plasma creatinine levels, minimum urinary output, minimum hematocrit, maximum plasma CRP, IL-6 or serum IDO levels (Table 11). There was no correlation between plasma cf-DNA and age (r=0.093, P=0.477). Furthermore, the plasma cf-DNA levels did not differ significantly between men and women (median 1.30 µg/ml, range 0.94-3.29 *vs* median 1.36 µg/ml, range 1.05-2.50, male *vs* female, P=0.303).

Table 11. The correlations of maximum plasma cell-free DNA with variables reflecting the severity of the disease in 61 patients with acute NE.

Variable	R	P-value
Duration of hospital stay	0.376	0.003
Platelets min	-0.297	0.020
Leukocytes max	0.388	0.002
Hematocrit min	-0.120	0.359
PTX3 max	0.513	<0.001
CRP max	-0.015	0.907
IL-6 max	0.202	0.168
IDO max	0.228	0.077
Creatinine max	0.101	0.436
Urinary output min	-0.063	0.636

Abbreviations: PTX3=pentraxin-3, CRP=C-reactive protein, IL-6=interleukin-6, IDO=indoleamine 2,3-dioxygenase, min=minimum, max=maximum

The maximum urinary excretion did not correlate with any of the variables reflecting disease severity, plasma cf-DNA level or age. The level of maximum urinary excretion did not differ between men and women (data not shown).

In the qualitative analysis of plasma cf-DNA, it was revealed that during the acute phase of the disease, cf-DNA showed a low-molecular weight pattern in most patients, corresponding to the size of apoptotic DNA fragments (150-200 bp) (IV,

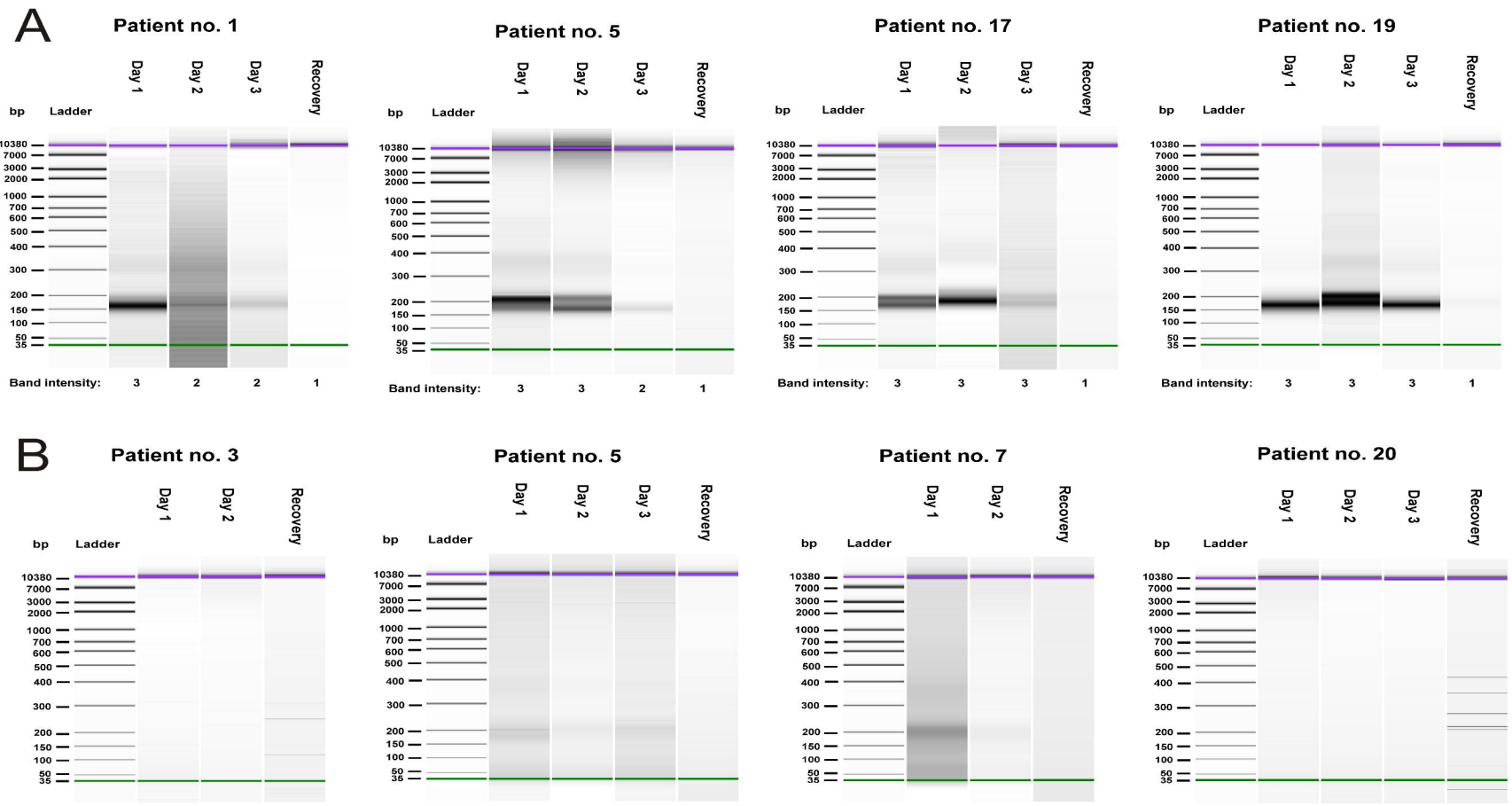
Figure 1A and 1B). This was observed independent of renal insufficiency. The visually graded maximum cf-DNA band intensity correlated positively with the maximum level of total plasma cf-DNA (r=0.513, *P*=0.021). However, the maximum cf-DNA band intensity did not correlate with any of the clinical or laboratory variables reflecting the severity of the disease. In control samples, taken four weeks after the release from the hospital, the low-molecular weight cf-DNA band was either absent or it was markedly weakened in all patients (IV, Figures 1A and 1B).

In the qualitative analysis of urine-cf-DNA, it was found that no distinguished low-molecular weight (150-200 bp) appearance of cf-DNA was detected during the acute phase of the disease, with the exception of two patients with renal insufficiency (patients No. 5 and 7) (IV, Figure 2). In addition, two patients (patients No. 3 and 20) had random-sized cf-DNA fragments in their convalescent phase urine samples (IV, Figure 2). In Figures 3A and 3B, the qualitative analysis data of plasma cf-DNA and of urine cf-DNA is shown from four patients as examples.

Figure legend for Figure 3 on page 78:

Figure 3. Qualitative analysis of plasma cf-DNA in 2 patients with maximum plasma creatinine >370 µmol/l (patients 1 and 5) and 2 patients with maximum plasma creatinine <125 µmol/l (patients 17 and 19) after NucleoSpin® Plasma XS kit extraction (A). Panel B shows qualitative analysis of urine cf-DNA in 3 patients with maximum plasma creatinine >370 µmol/l (patients 3, 5, and 7) and in a patient with maximum plasma creatinine <125 µmol/l (patient 20). Analyses were performed with Agilent's High Sensitivity Lab-on-a-chip DNA assay. Green lines indicate the low weight (35 bp) DNA marker and purple lines the high weight (10,380 bp) DNA marker. The intensity of low-molecular weight cf-DNA band was graded as follows: 1=no visible band or weak band intensity, 2=intermediate band intensity, 3=strong band intensity.

Figure 3.


A
Patient no. 1
Patient no. 5
Patient no. 17
Patient no. 19
Day 1
Day 2
Day 3
Recovery
bp
Ladder
10380
7000
3000
2000
1000
700
600
500
400
300
200
150
100
50
35
Band intensity: 3 2 2 1
Band intensity: 3 3 2 1
Band intensity: 3 3 3 1
Band intensity: 3 3 3 1
B
Patient no. 3
Patient no. 5
Patient no. 7
Patient no. 20

6. DISCUSSION

6.1 Clinical picture

The clinical picture of the disease was typical of NE in all Studies I-IV. In Study I, the patients were hospital-treated during the years 1997-2004, and 3 % of the patients suffered a clinical shock. In Studies II-IV, the patients were treated later, during the years 2000-2008, and none of the patients were in shock. Otherwise, the clinical course of the disease did not differ between the four studies as judged by the disease severity. All patients had fever and the median duration of fever was 5-6 days in Studies I-IV. It is noteworthy that in all studies the median duration of fever before admission to hospital was 4 days. Thus, the clinical and laboratory variables have been obtained median 4 days after the onset of the disease in all studies. Transient dialysis treatment was needed for 5-7 % of the patients in Studies I-IV. No deaths occurred in any of the studies and all patients recovered. Considering the typical laboratory findings of NE, the median of the maximum creatinine levels was 175-193 μmol/l in Studies I-IV. The median of the minimum platelet levels varied from 61 to 68 $x10^9$/l in Studies I-IV, while the lowest platelet level was less than 10 $x10^9$/l in all studies. The median of the maximum leukocyte counts and CRP levels were both moderately elevated in all Studies I-IV (9.9-10.1 $x10^9$/l and 69-80 mg/l, respectively). It should be noted that the highest leukocyte count as well as the highest CRP value in all studies (31.2 $x10^9$/l and 269 mg/l, respectively) were comparable to values seen in severe invasive bacterial infections Thus, these variables are not informative in clinical practice in differential diagnostics between PUUV and bacterial infections. All these findings are in concordance with previous studies concerning the clinical picture of NE (Settergren et al. 1989, Mustonen et al. 1994a, Braun et al. 2010).

6.2 C-reactive protein and pentraxin-3

Pentraxin family members are acute phase proteins produced in response to various inflammatory and infectious signals and they are considered as a part of the innate immune system (Garlanda et al. 2005). CRP, the prototype short pentraxin produced in the liver, was studied in Study I, whereas PTX3, the prototype long pentraxin produced at the site of inflammation, was studied in Study II.

Study I shows that the severity of acute NE is not associated with plasma CRP levels. The clinical or laboratory values reflecting the severity of the disease did not differ between patients having high or low CRP levels (>69 mg/l and ≤69 mg/l, respectively) with the exception of minimum urinary output. Furthermore, the occurrence of a pathologic chest radiograph had no significant association with high CRP levels. Instead, in multivariate analysis, high plasma CRP was found to be a protective factor against high serum creatinine (>193 μmol/l) (OR 0.3, 95 % CI 0.1−0.7, *P*=0.009) when examined together with age and high plasma IL-6. Age did not have an effect on high serum creatinine in this model, although the median age was higher in patients with high plasma CRP as compared to patients with low CRP levels.

Study I revealed that high CRP associates with preserved kidney function. This is the first report suggesting that CRP might act as a protective factor for kidney function in an infectious disease. Previously, it has been reported in a mouse model of SLE that administration of CRP prevents and reverses proteinuria and nephritis (Du Clos et al. 1994, Rodriguez et al. 2005, Rodriguez et al. 2006). It has also been shown that genetic factors associated with decreased CRP production predispose to SLE (Russell et al. 2004). These findings have been credited to the ability of CRP to enhance the clearance of immune complexes and to inhibit their accumulation in the renal cortex (Szalai et al. 2003). It should be noted that immune complexes have also been found in patients with PUUV infection (Penttinen et al. 1981). Reduced deposition and increased clearance of immune complexes could be the mechanism of high CRP to protect renal function in acute PUUV infection.

Previous studies concerning CRP and the severity or prognosis of viral infections have provided controversial results. In children, high CRP levels do not associate with a more severe disease in influenza or adenovirus infection (Appenzeller et al. 2002, Edelbauer et al. 2006). However, in influenza A H1N1pdm09 virus infection

and SARS, high CRP has been shown to be related to a more severe course of the disease (Wang et al. 2004, Zimmerman et al. 2010). Although in two studies concerning dengue virus infection, CRP levels associated with the severity of the infection, in another study carried out among Vietnamese children with dengue hemorrhagic fever, no association of CRP with disease severity was found (Bethell et al. 1998, Juffrie et al. 2001, Levy et al. 2010). In addition, high CRP has been shown as a predictor for a more severe clinical course of the disease and death in Crimean-Congo hemorrhagic fever (Yilmaz et al. 2010, Ozturk et al. 2012). Previous studies with negative results concerning CRP and its association with the prognosis of viral infections have been conducted with children. However, in Study I, no association of CRP with disease severity could be detected in acute PUUV infection in adults.

Study II shows that PTX3 levels are elevated during acute PUUV infection. The median maximum PTX3 level during the acute phase was 42.0 ng/ml, while during the convalescent phase the median PTX3 level was 1.1 ng/ml (P<0.001). In normal conditions, PTX3 blood level is <2 ng/ml in humans (Garlanda et al. 2005). In a study dealing with PTX3 in dengue virus infection, median PTX3 level was 80.8 ng/ml 2-3 days after the onset of symptoms (Mairuhu et al. 2005). Interestingly, the median of the maximum PTX3 levels was higher in patients with dengue virus infection and in Study II in patients with PUUV infection than in a previous study carried out with bacteremic patients (7.8 ng/ml) (Huttunen et al. 2011a). The reason for higher PTX3 levels in these viral infections as compared to bacteremic infections is unclear.

Study II also shows that high PTX3 levels are associated with a severe clinical course of acute PUUV infection and especially with thrombocytopenia. A maximum PTX3 level >101.6 ng/ml showed in ROC analysis a sensitivity of 71 % and a specificity of 89 % for detecting significant thrombocytopenia (platelet level <50 $x10^9$), with an AUC value of 0.78 (95 % CI 0.63–0.94). A high PTX3 level (>101.6 ng/ml) associated also with a higher maximum blood leukocyte count, plasma IL-6, creatinine and serum IDO values, as well as more severe anemia and longer hospital stay. Further, a high PTX3 level was associated with higher maximum hematocrit, lower minimum sodium level and greater change in body weight during hospitalization. It is noteworthy that hemoconcentration, fluid volume overload and

decreased sodium level are all considered signs of capillary leakage, which is the central pathogenetic feature in hantavirus infections.

There is only one previous clinical study concerning PTX3 in a viral infection. In dengue virus infection, PTX3 levels were found to be higher in patients with a severe form of the infection, dengue shock syndrome, compared to levels found in patients with milder diseases, dengue fever or dengue hemorrhagic fever (Mairuhu et al. 2005). Contrary to this clinical finding, in cell culture and in mouse models, PTX3 has been found to have antiviral activity against influenza virus and to protect from murine and human CMV infection (Bozza et al. 2006, Reading et al. 2008). Previous studies have also found that high PTX3 levels predict higher fatality in septicemia and septic shock (Mauri et al. 2010, Huttunen et al. 2011a, Vänskä et al. 2011). In Study II, a high PTX3 level was shown to associate with a more severe clinical course of acute PUUV infection, which is in concordance with the findings in dengue or septic patients.

In Study II, plasma PTX3 levels also correlated positively with terminal complement complex SC5b-9 and inversely with complement component C3 levels. Elevated SC5b-9 levels reflect the overall activation of the complement system, while C3 concentrations reflect the consumption during complement activation. Previously, it has been shown that complement activation is common in PUUV infection and the activation of both classical and alternative pathways has been associated with a severe disease (Paakkala et al. 2000, Sane et al. 2011). It has also been shown that PTX3 interacts with both the classical and alternative pathways of the complement system (Bottazzi et al. 1997, Deban et al. 2008). In Study II, it was shown that PTX3 levels correlate with the activation of the complement system and thrombocytopenia. These findings bring up the possibility that PTX3 is involved in the pathogenesis of thrombocytopenia in acute PUUV infection through the activation of the complement system. The activated complement cascade is able to activate the coagulation system and this in turn may lead to thrombocytopenia through the consumption of platelets (Peerschke et al. 2008). Supporting this idea, it has been shown that thrombocytopenia associates with decreased plasma anticoagulant levels, shortened thrombin time and enhanced fibrinolysis in acute PUUV infection (Laine et al. 2010). In Study II, the highest PTX3 levels were measured four days after the onset of fever, i.e. at the time of admission to the hospital simultaneously with the lowest platelet levels. Thereafter, PTX3 levels

declined and platelet levels rose. Possibly the PTX3 peak and the most severe thrombocytopenia occurred already before the admission to the hospital. Based on this analysis it is not possible to say, whether the peak PTX3 level preceded the most severe thrombocytopenia or not.

To conclude, the short pentraxin CRP does not reflect a clinically severe acute PUUV infection, whereas the long pentraxin PTX3 does. Moreover, CRP was shown to protect kidney function in Study I, possibly by reducing the deposition and enhancing the clearance of immune complexes. PTX3 in turn, associated strongly with thrombocytopenia and also with the activation of the complement system in Study II. PTX3 could act in the pathogenesis of thrombocytopenia in acute PUUV infection through the activation of the complement system. Finally, plasma PTX3 determinations offer a potential diagnostic tool for assessing the severity and outcome of acute NE, whereas CRP level is not informative in this viral infection.

6.3 Interleukin-6

IL-6 is a multifunctional cytokine and one of the most important cytokines responsible for the production of acute phase reactants, fever, and septic shock together with IL-1 and TNF-α.

Study I showed that high plasma IL-6 is associated with clinically severe acute PUUV infection. High maximum plasma IL-6 levels (>14.05 pg/ml) were found to associate with blood leukocytosis, thrombocytopenia, greater urinary protein excretion and change in body weight during hospitalization, lower minimum urinary output and systolic blood pressure, as well as with longer duration of hospitalization. In multivariate analysis, high IL-6 was found to be an independent risk factor for impaired renal function, thrombocytopenia, and longer hospitalization, when examined together with high CRP and age.

Previously, increased cytokine levels have been detected in plasma, urine, and various tissues of hantavirus infection patients (Linderholm et al. 1996, Temonen et al. 1996, Mori et al. 1999, Mäkelä et al. 2004). Concerning IL-6 in hantavirus infections, a Swedish study detected elevated IL-6 plasma levels in all patients with acute PUUV infection and found also a correlation between maximum levels of IL-6 and serum creatinine (Linderholm et al. 1996). Two Finnish studies have also found

IL-6 levels to be elevated in NE (Takala et al. 2000, Mäkelä et al. 2004). The first study found a negative correlation of serum IL-6 concentrations with mean arterial pressure and minimum platelet count (Takala et al. 2000). The second study found also urinary excretion of IL-6 to be increased. However, there was no correlation between plasma and urinary IL-6 levels, implying that IL-6 is possibly locally produced in the kidneys (Mäkelä et al. 2004). A German study detected significantly elevated levels of IL-6 in acute NE and the disease severity judged by elevated creatinine and low platelet counts correlated with high IL-6 levels (Sadeghi et al. 2011). Finally, a Brazilian study showed the levels of IL-6 to be elevated and to correlate with the severity of the disease in HCPS, and furthermore, very high IL-6 levels were seen in the fatal cases (Borges et al. 2008). In Study I, high IL-6 levels were found to associate with a severe clinical course of PUUV infection and also to be a risk factor for impaired renal function in concordance with previous findings in hantavirus infections. In other viral infections, studies concerning IL-6 and the severity of infection have produced controversial results relating high IL-6 levels to both more and less severe clinical diseases (Avila-Aguero et al. 2004, Bennett et al. 2007, Elliott et al. 2007, Bozza et al. 2008). This may be due to various factors, such as differences in the timing of sample collection and measuring disease severity.

The pathogenesis of hantavirus infections is currently not completely understood. Immunological factors, mainly abundantly expressed cytokines together with T cell activation, probably play an important role. More recent studies concerning cytokines in hantavirus infections have brought up the possibility that an imbalance in the production of pro-inflammatory and anti-inflammatory cytokines might be associated with the severity of the disease (Sadeghi et al. 2011, Saksida et al. 2011). In Study I, it was detected that the production of the pro-inflammatory cytokine IL-6 was associated with a clinically severe PUUV infection. However, other cytokines were not examined in this study.

To conclude, IL-6 is one of the most important pro-inflammatory cytokines. In the pathogenesis of hantavirus infections, cytokines probably play an important role and, possibly, an imbalance in the production of pro-inflammatory and regulatory cytokines is important. In Study I, it was shown that high IL-6 is associated with a clinically more severe acute PUUV infection. It was also shown that high IL-6 is an independent risk factor for renal insufficiency in acute PUUV infection. These

findings are in good agreement with previous findings concerning hantavirus infections.

6.4 Indoleamine 2,3-dioxygenase

IDO is an enzyme catalyzing the first and rate-limiting step in the catabolism of the essential amino acid tryptophan to kynurenine and its derivatives and thus limiting the availability of tryptophan (Mellor and Munn 2004, Mellor 2005). This leads to inhibition of microbial growth, but also inhibition of T cell responses and proliferation (Mellor and Munn 2004, Mellor 2005). Thereby IDO acts not only as a part of the innate immunity in antimicrobial defense, but also as an immunosuppressive agent.

Study III showed that IDO levels are elevated during acute PUUV infection. The median of the maximum IDO levels in the study was 199.3 µmol/mmol during the acute infection, while the median level in the convalescent phase was 64.7 µmol/mmol (P<0.001). In a previous Finnish study carried out in SLE patients, the median IDO level in healthy controls was 25.9 µmol/mmol (Pertovaara et al. 2007). In Study III, the IDO levels were lower during the convalescent phase than during the acute phase, but still higher than the values published for the healthy controls. Interestingly, in another Finnish study with bacteremic patients, the median of the maximum IDO levels was lower (89.9 µmol/mmol) than in Study III in patients with acute PUUV infection (Huttunen et al. 2010).

Study III also shows that high IDO levels are associated with increased disease severity of acute PUUV infection and especially renal impairment. A maximum IDO level >202 µmol/mmol showed in ROC analysis a sensitivity of 85 % and a specificity of 75 % for detecting maximum plasma creatinine levels >250 µmol/l and the AUC was 0.84 (95 % CI 0.76–0.91). A high IDO level, defined as a maximum IDO level >202 µmol/mmol, was found to be an independent risk factor for maximum serum creatinine level exceeding 250 µmol/l, when examined together with low urinary output, age, and sex. The other factors did not show significant association with significant renal insufficiency (creatinine>250 µmol/l) in this model. Thus, the finding of high IDO (kynurenine/tryptophan ratio) being a risk

factor for significant renal insufficiency is not explained by the possible decrease in the excretion of kynurenine. It was further detected that a high IDO level was associated not only with renal insufficiency, but also with higher blood leukocyte count and greater change in body weight during hospitalization, as well as with lower urinary output and blood hematocrit. It also associated with longer duration of hospitalization.

In previous studies dealing with IDO in viral infections, enhanced IDO activity has been detected in dengue virus infection and chronic HCV infection (Larrea et al. 2007, Becerra et al. 2009). Patients with chronic EBV infection or HIV infection have also been shown to have increased tryptophan degradation (Fuchs et al. 1990, Bellmann-Weiler et al. 2008). In the studies concerning dengue virus, HCV or EBV infection, the association between IDO level and the severity or progression of the disease was not studied. However, enhanced tryptophan degradation was also associated with progression of the disease and with complications, such as weight loss and neuropsychiatric disorders, in HIV (Fuchs et al. 1990, Schroecksnadel et al. 2007). Furthermore, antiretroviral therapy reduced tryptophan degradation (Fuchs et al. 1990). High IDO levels have also predicted severe disease and fatality in sepsis and septic shock (Huttunen et al. 2010, Tattevin et al. 2010). In concordance with the findings in HIV infection and sepsis, a high IDO level was associated with increased severity of PUUV infection in Study III.

Concerning renal diseases, it has previously been demonstrated that the levels of tryptophan metabolites increase in chronic renal insufficiency apparently not due to a decrease in renal excretion, but due to an increase in production and/or decrease in degradation (Saito et al. 2000, Schefold et al. 2009). In mice, it has been shown that IDO enhances renal ischemia-reperfusion injury (Mohib et al. 2008). On the contrary, in a mouse model of crescentic glomerulonephritis, IDO acts as a protective factor (Hou et al. 2009). The latter finding might be explained by the immunosuppressive function of IDO. In Study III, a high IDO level was strongly associated with renal impairment in acute PUUV infection.

Whether IDO is merely a marker of severe disease in PUUV infection, or has a pathogenetic role in it, is not known. However, it is possible that IDO is involved in the pathogenesis of renal failure in NE. It has previously been demonstrated that increased IDO activity promotes TEC apoptosis and inhibition of IDO enhances TEC survival (Mohib et al. 2007). In Study III, it was found that high IDO was

strongly associated with significant renal failure. IDO levels were also found to peak before creatinine levels, which supports the idea of IDO being involved in the pathogenesis of renal failure in acute PUUV infection. The pathogenetic mechanism could be promotion of TEC apoptosis. Noteworthy is that signs of epithelial cell apoptosis have previously been detected in patients with PUUV infection (Klingström et al. 2006). Another pathogenetic mechanism could be enhancement of the infection by inducing immunosuppression through T cell suppression and apoptosis.

Taken into account its effects on T cells, IDO is an interesting enzyme in the context of hantavirus infections. Although the pathogenesis of hantaviral infections is currently still not completely understood, immunological factors, especially T cell activation together with cytokines probably play an important role. It has been suggested that virus-specific CD8+ cells are important in the development of endothelial cell dysfunction and capillary leakage in hantavirus infections (Terajima et al. 2007). In kidney biopsies of patients with NE, there is an increased amount of infiltrating cells in the peritubular areas including plasma cells, monocyte/macrophages, polymorphonuclear leukocytes, as well as lymphocytes, predominantly CD8+ T cells (Temonen et al. 1996). It has also been shown that the severity of the histopathological changes is associated with the clinical severity of renal failure. These findings are indicative of an important role of cell-mediated cytotoxicity in the pathogenesis of renal failure in PUUV infection.

To add up, IDO has multiple functions in the immune system. It has an inhibitory effect on T cells, which have an important role in the pathogenesis of hantavirus infections. In the present study, it was shown that a high IDO level associates with clinically severe acute PUUV infection and especially with the severity of renal insufficiency. IDO may have a pathogenetic role in the development of renal failure in PUUV infection through its enhancive functions on TEC apoptosis or inhibitory functions on T cells.

6.5 Cell-free DNA

The current view is that, in clinical conditions, cf-DNA originates from apoptotic or necrotic cells and therefore its plasma levels reflect the amount of cellular damage

(Jahr et al. 2001). Hantaviruses have been considered noncytopathic (Yanagihara et al. 1985, Temonen et al. 1993), although under certain conditions Tula hantavirus induces apoptosis in cultured cells (Li et al. 2004, Li et al. 2005). Also, a Swedish study in PUUV-infected patients showed that epithelial cell apoptosis is induced during the acute infection (Klingström et al. 2006).

Study IV showed that plasma cf-DNA levels are elevated during acute PUUV infection, but the urinary excretion of cf-DNA is not. The median of the maximum cf-DNA levels during the acute phase was 1.33 µg/ml, while during the convalescent phase, the median cf-DNA level was 0.77 µg/ml (P<0.001). In a previous study with bacteremic patients, the median of the maximum cf-DNA levels was 2.03 µg/ml in nonsurvivors and 1.26 µg/ml in survivors (Huttunen et al. 2011b). In Study IV, the median of the maximum plasma cf-DNA levels in acute PUUV infection equals with the level seen in survived bacteremia patients. The urinary excretion of cf-DNA was not increased during the acute phase compared to urinary excretion during the convalescent phase.

Study IV also showed that plasma cf-DNA level correlates with the severity of acute PUUV infection, whereas urine cf-DNA excretion does not. The total plasma cf-DNA level and blood platelet count showed a negative correlation. In addition, plasma cf-DNA levels correlated positively with blood leukocyte count and the length of the hospital stay. The latter is probably the best variable reflecting the overall severity of the disease. Plasma cf-DNA levels also correlated with plasma PTX3 levels. This is explained by the fact that PTX3 contributes to the opsonization and clearance of apoptotic and necrotic cells (Bottazzi et al. 2009), which are regarded as the origin of cf-DNA. There was no statistically significant correlation between plasma cf-DNA and IDO levels, despite the fact that IDO promotes apoptosis of TECs. The total plasma cf-DNA level did not correlate with the severity of renal insufficiency. Moreover, maximum urine cf-DNA secretion did not correlate with any clinical or laboratory variables reflecting the severity of the infection. Neither did maximum urine cf-DNA excretion correlate with maximum plasma cf-DNA levels.

In the qualitative analysis of plasma cf-DNA, it was revealed that, during the acute phase of the disease, cf-DNA displayed a low-molecular weight pattern in most patients, corresponding to the size of apoptotic DNA fragments (150-200 bp). After recovery, such cf-DNA pattern was not observed. It is therefore plausible that

the detected low-molecular weight cf-DNA originated from apoptotic cells during the acute phase of the disease. An earlier study detecting signs of epithelial cell apoptosis during acute PUUV infection supports this idea (Klingström et al. 2006). Furthermore, Study IV showed that the maximum total plasma cf-DNA concentration and the apoptotic cf-DNA band intensity correlated, which also supports the hypothesis that the increase in plasma cf-DNA is due to apoptosis. Similar results concerning apoptotic cf-DNA pattern have been observed also in bacteremic patients (Huttunen et al. 2011b). However, in Study IV, the plasma cf-DNA apoptotic band intensity was not associated with the severity of PUUV infection. It should be noted, however, that the qualitative analysis was performed only for twenty patients. This may have been too small a sample size to detect possible correlations between the band intensity and the disease severity reflecting variables.

In the qualitative analysis of urine cf-DNA, no low-molecular weight pattern of cf-DNA that could be attributed to the disease severity was detected. However, two patients with renal insufficiency had a clear low-molecular weight cf-DNA band in their urine during the acute phase of the disease, possibly indicating increased apoptosis in the renal system.

In viral infections, previous studies concerning plasma cf-DNA are scarce. In patients with occult HBV infection, the levels of cf-DNA have been detected to be elevated (Bhargava et al. 2010). In a recent study, the cf-DNA levels were elevated and they correlated with the severity of the infection in dengue patients (Ha et al. 2011). Also, in septic patients, elevated plasma cf-DNA levels have predicted poor clinical outcome (Zeerleder et al. 2003, Rhodes et al. 2006, Saukkonen et al. 2008, Huttunen et al. 2011b). In agreement with previous studies in patients with dengue virus infection or sepsis, Study IV showed that, in acute PUUV infection, plasma cf-DNA levels are elevated and they correlate with the severity of the disease, although not with the degree of renal insufficiency.

The urinary excretion of cf-DNA did not correlate with the severity of acute PUUV infection in Study IV, whereas total plasma cf-DNA did. This discrepancy between the correlations of urine and plasma cf-DNA suggests that the cf-DNA detected in urine may not be clinically relevant and does not reflect the degree of inflammation in the kidneys. Previous studies concerning urinary cf-DNA support these findings. In hematopoetic stem cell transplant patients, the quantity of donor-

derived DNA does not correlate with the quantity of plasma cf-DNA and the predominant cf-DNA fragment size also differs between plasma and urine (Hung et al. 2009). A similar DNA fragment size difference between plasma and urine has also been detected in pregnant women carrying male fetuses (Koide et al. 2005). Previously, cf-DNA has been shown to be elevated in the urine of patients with urinary tract infection (Garcia Moreira et al. 2009). On the other hand, in colorectal cancer patients, urine secreted cf-DNA contains tumor-derived K-ras mutations (Su et al. 2004, Su et al. 2005). In addition, fetal Y-chromosomal DNA sequences have been found in the urine of pregnant women carrying male fetuses (Koide et al. 2005, Shekhtman et al. 2009). These findings indicate that urinary cf-DNA probably consists of a heterogeneous mixture of cf-DNA fragments originating from dying cells in the renal system and from the pool of plasma cf-DNA. It is known that cf-DNA can cross the kidney barrier (Botezatu et al. 2000). However, the exact mechanism by which it crosses the glomerular basement membrane is unclear. The maximum urinary excretion of cf-DNA has been thought to be influenced by renal function. However, the results of Study IV do not support this hypothesis since no correlation between urinary secretion of cf-DNA and renal function was detected.

To conclude, the elevated levels of cf-DNA in different diseases originate from apoptotic or necrotic cells. In Study IV, it was shown that the maximum total plasma cf-DNA level is elevated during acute PUUV infection and it correlates with the severity of the disease, although not with the degree of renal insufficiency. It was also shown that the maximum total plasma cf-DNA level correlates with the apoptotic cf-DNA band intensity, suggesting that the increase in plasma cf-DNA is due to apoptosis. This finding supports the previous findings indicative of epithelial cell apoptosis in acute PUUV infection. The urinary excretion of cf-DNA, in turn, does not reflect the degree of inflammation in the kidneys.

6.6 Future considerations

In the present study, CRP, PTX3, IL-6, IDO, and cf-DNA were analyzed in acute PUUV infection as markers of disease severity, but also their possible pathogenetic role in the disease was evaluated. The precise mechanisms in the pathogenesis of PUUV as well as other hantavirus infections are still not completely understood.

Central clinical findings in acute NE are thrombocytopenia and acute renal insufficiency together with the vascular permeability syndrome.

Thrombocytopenia is a characteristic phenomenon of NE, causing sometimes even fatal bleedings. In Study II, it was revealed that high plasma PTX3 levels associate strongly with thrombocytopenia as well as with the activation of the complement system. It is postulated that PTX3 might be involved in the pathogenesis of thrombocytopenia in acute NE through the complement system, which in turn would activate the coagulation system and lead to the consumption of platelets. In concordance with this idea are the findings that thrombocytopenia associates with decreased plasma anticoagulant levels, shortened thrombin time and enhanced fibrinolysis in NE (Laine et al. 2010). It has been suggested that the interaction of platelets with endothelium, their activation, and P-selectin expression could provide mechanisms of thrombocytopenia during hantavirus infection (Laine et al. 2010). Furthermore, it has been proposed that enhanced platelet adhesion and activation could result in platelet consumption and thrombocytopenia (Laine et al. 2011). However, further studies concerning platelets and the coagulation system are needed to establish the exact mechanisms of thrombocytopenia in HFRS. Moreover, the association of PTX3 with other elements of the coagulation system have not been studied in hantavirus infections. Studies elucidating the association of PTX3 with different elements of the coagulation system might clarify the role of PTX3 in the development of thrombocytopenia in HFRS.

In Study III, high IDO was identified as a predictive factor for severe clinical course of NE and as an independent risk factor for significant renal insufficiency. Increased IDO activity is known to promote TEC apoptosis and this could provide a possible pathogenetic mechanism for the development of renal failure in NE (Mohib et al. 2007). However, IDO is also known to inhibit T cell responses and proliferation and thus lead to immunosuppression. Therefore, IDO could be involved in the pathogenesis of NE also via impaired cellular immunity. Furthermore, CD8+ T cells are considered important in the pathogenesis of hantavirus infection including the capillary leakage syndrome. In addition, an imbalance between effector T cells and Tregs possibly has a role in the pathogenesis. Noteworthy is that IDO also inducts Tregs. Future studies concerning the role and significance of Tregs in hantavirus infections are needed. Moreover, the

association of IDO and T cell functions in hantavirus infection require further studies in order to establish the role of IDO in the infection.

Capillary leakage is the central phenomenon in the pathogenesis of hantavirus infection explaining many clinical signs and symptoms of the disease. However, the mechanisms behind this phenomenon remain currently still to be elucidated. VEGF is an interesting factor quite recently studied in the context of hantavirus infections. It has been demonstrated that pathogenic hantaviruses enhance the permeability of endothelial cells in response to VEGF (Gavrilovskaya et al. 2008). Moreover, hantavirus-directed permeability has been inhibited by antibodies to VEGF2 (Gavrilovskaya et al. 2008). Antibodies to VEGF as well as VEGF2 are already commercially available and in clinical use in oncology. These agents could offer a therapeutic possibility also in hantavirus infections. However, this type of therapy has shown common adverse vascular effects that may limit its clinical use (Hayman et al. 2012). Nevertheless, further clinical studies concerning VEGF and its role in the pathogenesis of capillary leakage syndrome in hantavirus infections are warranted.

Finally, there were no control patient groups in any of the studies in this thesis. In further studies, control individuals with other viral infections would help to clarify the specificity of the findings to PUUV/hantavirus infection instead of more generally to viral infections.

7. SUMMARY AND CONCLUSIONS

The association of CRP, PTX3, IL-6, IDO, and cf-DNA with the severity of acute PUUV infection, and their possible role in the pathogenesis of NE can be summarized as follows:

I High plasma CRP level is not associated with a severe clinical course of NE. On the contrary, high CRP level was revealed as a possible protective factor against renal failure.

II PTX3 level is elevated during acute NE. High plasma PTX3 level is associated with a more severe course of NE and especially thrombocytopenia. In addition, PTX3 level associates with the activation of the complement system. These findings bring up the possibility that PTX3 is involved in the pathogenesis of thrombocytopenia in NE through the complement system.

III High IL-6 level is associated with clinically severe NE. High IL-6 is also an independent risk factor for impaired renal function in NE.

IV IDO is upregulated during acute NE. A high IDO level is associated with clinically severe acute NE and, most of all, impaired renal function. IDO is an independent risk factor for significant renal insufficiency and may be involved in the pathogenesis of renal failure either through its effects on TECs or T-cells.

V Total plasma cf-DNA level is elevated during acute NE and it associates with the severity of NE, but not with the degree of renal insufficiency. Furthermore, the plasma cf-DNA level and the apoptotic band intensity correlate, indicating that the increase in plasma cf-DNA is likely due to cellular apoptosis. The urinary excretion of cf-DNA, in turn, is not increased during acute NE and does not

correlate with the severity of the disease indicating that the urinary excretion of cf-DNA does not reflect the degree of inflammation in the kidneys.

In conclusion, high plasma PTX3, IL-6, cf-DNA, and serum IDO levels are associated with a severe clinical course of NE, although cf-DNA is not associated with the severity of renal failure. PTX3 correlates most of all with thrombocytopenia. Since it also correlates with the activation of the complement system, it is possible that PTX3 is involved in the pathogenesis of thrombocytopenia in NE through the activation of the complement cascade. IDO activity, in turn, is associated especially with significant renal insufficiency. As IDO is known to promote TEC apoptosis, it is possible that it is involved in the pathogenesis of renal impairment in NE through TEC apoptosis. Furthermore, the suppressive effect of IDO on T cells may induce immunosuppression and promote renal insufficiency by this mechanism. The results of the qualitative analysis of plasma cf-DNA indicate that apoptosis occurs during acute NE. Urinary excretion of cf-DNA, however, does not reflect the degree of inflammation in the kidneys. Finally, high CRP does not indicate a clinically severe NE. On the contrary, it may protect the kidneys, possibly by enhancing the clearance of immune complexes.

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