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PAULA MANTULA

Role of Urinary Findings and Adipokines in Puumala Virus-induced Acute Kidney Injury

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Role of Urinary Findings and Adipokines in Puumala Virus-induced Acute Kidney Injury

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To my family and my beloved brother.

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Tampere, February 2021
Paula Mantula

ABSTRACT

Acute kidney injury (AKI) triggered by various factors is one of the common complications in hospital-treated patients, often leading to a prolonged need of hospital care. Although predisposing factors can be apparent, the pathogenesis of AKI remains unclear. AKI is considered as one of the factors related to worse prognosis in many disease states. Biomarkers to predict severe AKI are scarce and specific treatment for AKI does not exist. Hemorrhagic fever with renal syndrome (HFRS), caused by Puumala virus (PUUV), also called nephropathia epidemica, is a common cause of AKI in Finland. The clinical course of PUUV infection varies from mild, even asymptomatic, to severe. The majority of patients admitted to hospital due to acute PUUV infection have AKI, severe AKI in a third of them. However, AKI related to PUUV infection has a favourable prognosis and renal recovery is usually complete. While the clinical course of PUUV infection is well known, the pathogenesis of infection is only partly understood.

A high amount of abruptly emerging proteinuria is a usual finding in acute PUUV infection. The association between the amount of albuminuria and disease severity was analysed in 205 patients with acute PUUV infection. Higher degree of albuminuria detected by urinary dipstick tests on hospital admission were associated with more severe upcoming AKI during hospital stay. The amount of albuminuria also correlated with other markers reflecting disease severity. Peak values of urinary protein excretion were detected 4-5 days earlier than the peak values of plasma creatinine. Higher degree of haematuria in urinary dipstick tests on hospital admission was associated with higher degree of dipstick-verified albuminuria and also with the severity of AKI during hospitalization.

Glucosuria in urinary dipstick tests was detected in 12 % of 195 patients with acute PUUV infection on hospital admission. The presence of glucosuria was associated with all markers of disease severity including the severity of AKI, lower blood thrombocyte count and the presence of clinical shock, a sign of severe capillary leakage in acute PUUV infection. The presence of glucosuria was not explained solely by high blood glucose but maybe a transient change in tubular function.

Albuminuria has been found to be transient in PUUV infection, although reports about the disappearance rate are missing. Urinary albumin excretion in various time-

points during the acute and convalescent phase within six months after acute infection was studied in 141 patients to discover the rate of decrease. Albuminuria had disappeared within 2-3 weeks after fever onset. Disappearance rate was not affected by the amount of albuminuria, or the severity of AKI during the acute phase of the disease.

In acute PUUV infection, a strong immune activation is detected. Adipokines, also called adipocytokines, are considered to have an immunomodulating effect. The levels of plasma adipokines (resistin, leptin and adiponectin) were determined in the acute phase, convalescent phase and after one year in 79 patients with acute PUUV infection. Resistin levels were clearly elevated during acute infection compared with levels determined later. Higher resistin levels correlated with the severity of AKI and also a higher degree of dipstick albuminuria on hospital admission. The slight changes in plasma levels of other adipokines did not have significant associations with the disease-severity markers.

To conclude, the probability of severe AKI emerging in acute PUUV infection can be assessed by combining the plus-counts of urinary dipstick test results for albuminuria, haematuria and glucosuria detected in the early phase of the disease. Such correlation of urinary findings with AKI severity have not been reported previously in any form of AKI. The presence of glucosuria can be a warning sign of a severe life-threatening outcome as it was associated with clinical shock. Of note, the delay between urine testing and fever onset should be taken into account, as the alterations in urine findings are rapid and thus delays affect predictive capacity of these urine tests. Some of the markers of host inflammatory response are associated with disease severity in acute PUUV infection. The rise in plasma resistin level, correlates with the severity of AKI and higher degree of albuminuria in hospital-treated PUUV-infected patients.

TIIVISTELMÄ

Akuutin munuaisvaurion (acute kidney injury, AKI) laukaisevia tekijöitä on useita ja se on yksi yleisimmistä sairaalapotilaiden komplikaatioista, joka johtaa hoidon tarpeen pitkittymiseen. Vaikka AKI:n laukaiseva tekijä on usein pääteltävissä, sen patogeneesi on tuntematon ja sen tiedetään liittyvän huonoon ennusteeseen monissa sairaustiloissa. Biomarkkereita, joilla vaikea AKI olisi ennustettavissa, ei juuri tunneta eikä akuuttiin munuaisten vajaatoimintaan ei ole spesifistä hoitoa. Puumala-viruksen aiheuttama myyräkuume on Suomessa yleinen AKI:n aiheuttaja. Infektion vaikeusaste vaihtelee lievästä, jopa oireettomasta, vakavaan tautimuotoon. Sairaalahoitoon joutuneista myyräkuumepotilaista valtaosalla todetaan AKI ja kolmasosalla se on vaikea-asteinen. Myyräkuumeeseen liittyvän AKI:n ennuste on kuitenkin hyvä ja munuaisilmentymien tiedetään korjaantuvan yleensä täydellisesti. Vaikka myyräkuumeen taudinkulku tunnetaan hyvin, sen patogeneesi tunnetaan vain osittain.

Runsas, nopeasti ilmaantuva proteinuria on myyräkuumeessa tavallista. Albuminurian määrän yhteyttä taudin vaikeusasteeseen selvitettiin 205 myyräkuumepotilaalla. Mitä enemmän albuminuriaa sairaalaan tulovaiheessa todettiin virtsan liuskatestillä, sitä vaikeampi AKI kehittyi sairaalahoidon aikana. Albuminurian määrä korreloi myös muihin muuttujiin jotka kuvastavat taudin vaikeusastetta. Virtsan proteiinien erityksen huippu todettiin 4-5 päivää aiemmin kuin korkeimmat plasman kreatiniiniarvot. Myös sairaalaan tulovaiheen virtsan liuskatestin haematurian määrän todettiin näillä 205 potilaalla liittyvän vaikeampaan munuaisvaurioon sairaalahoidon aikana.

Glukosuriaa havaittiin sairaalaan tullessa virtsan liuskatestissä 12 %:lla 195 myyräkuumepotilaasta. Sen ilmenemisellä todettiin olevan vahva yhteys kaikkiin taudin vaikeusastetta kuvaaviin muuttujiin, kuten munuaisvaurion vaikeusasteeseen, verihiutaleiden mataluuteen ja sokkioireisiin, jotka liittyvät myyräkuumeessa vaikeaan kapillaarisuonten läpäisevyyshäiriöön. Glukosuria ei selittynyt selvästi korkeilla veren sokeriarvoilla, vaan sen taustalla lienee ohimenevä tubulusfunktion häiriö.

Albuminurian on todettu olevan ohimenevää myyräkuumeessa, mutta sen häviämisnopeudesta ei ole julkaistu tutkimustietoa. Albuminurian häviämisnopeutta ja siihen vaikuttavia tekijöitä tutkittiin 141 myyräkuumepotilaalla eri ajankohdissa,

sekä akuutissa vaiheessa, että toipumisvaiheessa 6 kuukauden kuluessa infektiosta. Albuminuria todettiin hävinneeksi 2-3 viikon kuluessa kuumeen alusta eikä häviämisen nopeus riippunut sen määrästä tai AKI:n vaikeusasteesta taudin akuutissa vaiheessa.

Puumala-virusinfektion aikana elimistössä todetaan voimakas immuunivasteen aktivoituminen. Adipokiinit, joita kutsutaan myös adiposytokiineiksi, osallistuvat inflammaatiovasteen säätelyyn. Plasman adipokiinien (resistiini, leptiini ja adiponektiini) pitoisuudet määritettiin 79 potilaalta taudin akuutissa ja toipumisvaiheessa, sekä vuoden kuluttua taudin sairastamisesta. Resistiinin todettiin olevan selvästi koholla taudin akuutissa vaiheessa verrattuna myöhempiin mittauksiin. Resistiinin nousu korreloi AKI:n vaikeuteen. Korkeampi resistiinitaso korreloi myös suurempaan albuminurian määrään sairaalaan tulovaiheen liuskatestissä. Muiden adipokiinien lievillä plasmapitoisuuksien muutoksilla ei ollut selvää yhteyttä myyräkuumeen vaikeusasteeseen.

Yhteenvetona todetaan, että myyräkuumeessa voidaan arvioida vaikean munuaisvaurion kehittymisen riskiä laskemalla taudin varhaisessa vaiheessa otetun virtsan liuskatestin albuminurian, hematurian ja glukosurian määrät yhteen. Tällaista virtsalöydösten yhteyttä akuutin munuaisvaurion vaikeusasteeseen ei ole aiemmin raportoitu missään muissa AKI:n muodoissa. Glukosurian esiintyminen varoittaa myös suuremmasta riskistä henkeä uhkaavalle taudille, koska se liittyi kliinisen sokin esiintymiseen. On syytä huomioida, että virtsalöydösten muutokset ovat myyräkuumeessa nopeita, joten tulkinnassa täytyy ottaa huomioon viive kuumeen alusta näytteenottohetkeen. Joillakin inflammaatiovasteen markkereista on todettu olevan yhteyttä myyräkuumeen vaikeusasteeseen. Kohonnut plasman resistiini-taso liittyy sekä munuaisvaurion vaikeusasteeseen että albuminurian määrään sairaalahoitoa vaativassa myyräkuumeessa.

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ABBREVIATIONS

AKI	acute kidney injury
ANDV	Andes virus
ATIN	acute tubulointerstitial nephritis
AUROC	area under the receiver operating characteristic curve
BAL	bronchoalveolar lavage
BMI	body mass index
CI	confidence interval
CKD	chronic kidney disease
CNS	central nervous system
CRP	c-reactive protein
CTL	cytotoxic T lymphocytes
cU-Alb	timed urinary excretion of albumin
cU-IgG	timed urinary excretion of immunoglobulin G
cU-1miglo	timed urinary excretion of α_1-microglobulin
DOBV	Dobrava virus
ECG	electrocardiogram
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FSGS	focal segmental glomerulosclerosis
GATA-3	type-2 cytokine transcription factor
GFR	glomerular filtration rate
HCPS	hantavirus cardiopulmonary syndrome
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HLH	haemophagocytic lymphohistiocytosis
HPS	hantavirus pulmonary syndrome
HRCT	high-resolution computed tomography
HTNV	Hantaan virus
ICU	intensive care unit

Ig	immunoglobulin
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
INF	interferon
INS	idiopathic nephrotic syndrome
MRI	magnetic resonance imaging
NET	neutrophil extracellular traps
NGAL	neutrophil gelatinase-associated lipocalin
NK	natural killer
OR	odds ratio
PTX3	pentraxin-3
PUUV	Puumala virus
ROC	receiver operating characteristic
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SC5b-9	membrane attack complex of the complement system
SEOV	Seoul virus
SGLT-2/GLUT2	sodium-glucose cotransporter type 2/glucose transporter 2
sICAM-1	soluble intercellular adhesion molecule 1
SNV	Sin Nombre virus
suPAR	soluble cell-membrane protein urokinase-type plasminogen activator receptor
sVCAM-1	soluble vascular adhesion molecule 1
TINU	tubulointerstitial nephritis and uveitis
TNF	tumor necrosis factor
U-Alb-stick	urinary dipstick test for albumin
U-Eryt-stick	urinary dipstick test for erythrocytes
U-Gluk-stick	urinary dipstick test for glucose
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

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II Outinen T, Mantula P, Laine O, Pörsti I, Vaheri A, Mäkelä S, Mustonen J. Haematuria is a marker for the severity of acute kidney injury but does not associate with thrombocytopenia in acute Puumala hantavirus infection. Infect Dis (London) 2017; 49:840-846. https://doi.org/10.1080/23744235.2017.1358461.

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IV Mantula P, Tietäväinen J, Clement J, Niemelä O, Pörsti I, Vaheri A, Mustonen J, Mäkelä S, Outinen T. Flash-like albuminuria in acute kidney injury caused by Puumala hantavirus infection. Pathogens 2020;9(8), 615. https://doi.org/10.3390/pathogens9080615.

V Mantula P, Outinen T, Jaatinen P, Hämäläinen M, Huhtala H, Pörsti I, Vaheri A, Mustonen J, Mäkelä S. High plasma resistin associates with severe acute kidney injury in Puumala hantavirus infection. PlosOne 2018 13(12): e0208017. https://doi.org/10.1371/journal.pone.0208017.

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1 INTRODUCTION

Puumala virus (PUUV) belongs to the diverse hantaviridae virus family and is the most common hantavirus in Europe. Hantaviruses are present in all continents of the world [1, 2]. Every hantavirus has its own natural carrier animal, usually small mammals like rodents, which in the case of PUUV is the bank vole, *Myodes glareolus* [3]. In humans, zoonotic infection by PUUV manifests as a mild hemorrhagic fever with renal syndrome (HFRS) also called nephropathia epidemica. The syndrome was first described in Scandinavia in case reports during the 1950s to 1970s [4]. Since then, in Scandinavia, the European part of Russia and some central European countries, thousands of annual infections are consistently reported, with the highest incidence and prevalence of population seropositivity in Finland [5-8].

The course of HFRS caused by PUUV varies from mild and asymptomatic to a severe outcome. The symptoms of illness include fever, headache, abdominal and back pains, nausea and fatigue. Renal involvement, acute kidney injury (AKI) with proteinuria and haematuria, and thrombocytopenia are the hallmarks of PUUV infection [9]. Increased capillary endothelial permeability resulting in plasma leakage from the vasculature into tissues is a phenomenon manifested with varying severity in the early phase of the disease. It can result in haemoconcentration, fluid accumulations in various tissues and sometimes hypotension and shock symptoms as the effective blood volume decreases [10]. AKI is frequently observed in patients admitted to hospital whereas severe bleeding is rare, even if blood thrombocyte counts are found to be clearly lowered [11]. Although AKI is detected in the majority of hospitalized patients and regarded as severe in 30 % of patients, the recovery of kidney function is usually complete without any sequelae. A transient need of hemodialysis treatment occurs in 4 % of hospital-treated Finnish patients [12]. In rare severe life-threatening disease outcomes, signs of circulatory shock, pulmonary oedema and severe bleeding can occur. The mortality rate in PUUV infection is under 0.1 % in Finland [13]. Treatment of PUUV infection is supportive, as no specific treatment exists.

Renal involvement in acute PUUV infection is characterized by oliguric AKI with abrupt onset of proteinuria. Proteinuria is detected in over 90 % of patients in the

early phase of the disease. The amount of proteinuria is notable, exceeding 3500 mg/d in 25 % of the patients. A rapid decrease in the amount of proteinuria have been documented in a few cases during hospital stay, but the disappearance rate has not been systemically studied [14]. Microscopic haematuria is present in over 70 % of patients with acute infection [11, 15]. Renal histological findings in light microscopy are characterized as acute tubulointerstitial nephritis (ATIN) with interstitial oedema and inflammatory-cell infiltrates in the interstitium. Medullary haemorrhages are typical findings, but also microvascular inflammation and acute tubular necrosis (ATN) of varying extent have been reported [16, 17]. Compared with other ATINs with varying etiology, the amount of proteinuria in PUUV infection is exceptionally high.

The host inflammatory response is considered important, explaining the varying severity of the disease in PUUV infection. Both innate and cellular immune responses are observed in peripheral blood and various organs also other than the kidneys. Cytokines, complement activation, cytotoxic T cells and monocyte macrophages are considered to play a central role in the pathogenesis of the disease [18, 19]. The trigger of sudden capillary leakage and proteinuria distinguishing PUUV infection from many other infections is as yet unexplored. However, other kidney diseases with sudden nephrotic-range proteinuria are not typically presented with AKI.

The purpose of this study was to evaluate the possible associations of urinary findings with disease severity in acute PUUV infection. After PUUV-induced AKI with exceptionally good recovery of kidney function, long-standing albuminuria is not typical. To further assess the disappearance rate and to clarify possible factors affecting it, albuminuria at different time-points soon after acute PUUV infection was studied. In addition, as novel inflammatory markers, plasma adipokine changes in PUUV infection and their associations with disease severity were addressed.

2 REVIEW OF THE LITERATURE

2.1 Puumala virus and other hantaviruses

2.1.1 Virology

Puumala virus (PUUV) is a single-stranded, enveloped, negative-sense RNA virus in the family Hantaviridae of the order Bunyavirales. Members of this genus are called orthohantaviruses or simply hantaviruses. In what follows, the latter designation is used. The hantavirus family now contains over forty recognized viruses [1]. Hantaviruses are carried by small mammals and are present in almost every continent of the world [20]. Each virus has its specific host species such as moles, mice, bats or shrews which are considered to be chronic, asymptomatic carriers whose excreta contains the virus. The human disease-causing ability of several viruses in the hantavirus family is not known, since evidence of infection concerning some viruses is based only on human serological findings, without clearly identified symptoms of infection [21]. Hantavirus infection in humans is a zoonosis and usually not contagious between humans [22]. However, the few cases of hantaviral disease caused by Andes virus (ANDV), are considered to be transmitted from human to human in South-America [23].

As new hantavirus species are constantly being found and genomic sequencing data is expanding, the classification and taxonomic modification of the current hantavirus family is changing [1]. One of the latest lists of hantaviruses with the ability to cause clinical infection in humans, and their global distributions, are presented in Table 1 and Figure 1 [20]. The total hantaviral disease burden, especially in the developing countries, remains unknown.

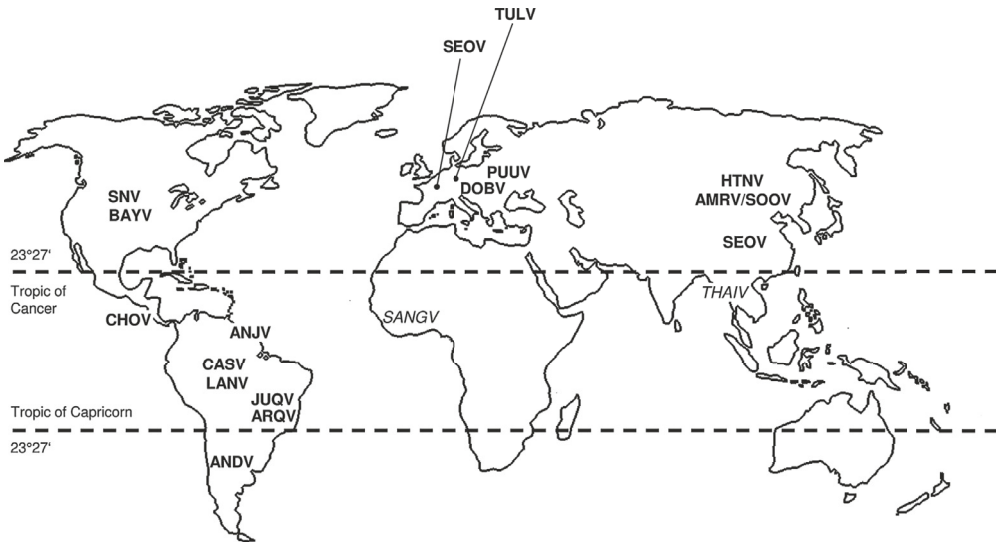
Table 1. Hantaviruses with evidence of human pathogenicity according to the type of human disease and geographic distribution

Americas	HCPS	Africa	HFRS	Europe	HFRS
SNV	Sin Nobre virus	*SANGV*	*Sangassou virus*	PUUV	Puumala virus
BAYV	Bayou virus			DOBV	Dobrava virus
CHOV	Choclo virus			SEOV	Seoul virus
ANJV	Anajatuba virus	Asia	HFRS	*TULV*	*Tula virus*
ANDV	Andes virus	HTNV	Hantaan virus		
CASV	Castelo dos Sonhos virus	AMRV/SOOV	Amur/Soochong virus		
LANV	Laguna Negra virus	SEOV	Seoul virus		
JUQV	Juquitiba virus	*THAIV*	*Thailand virus*		
ARQV	Araraquara virus				

HCPS: Hantavirus Cardiopulmonary Syndrome; HFRS: Haemorrhagic Fever with Renal Syndrome

Italic: viruses with uncertain human pathogenicity (serological findings only)

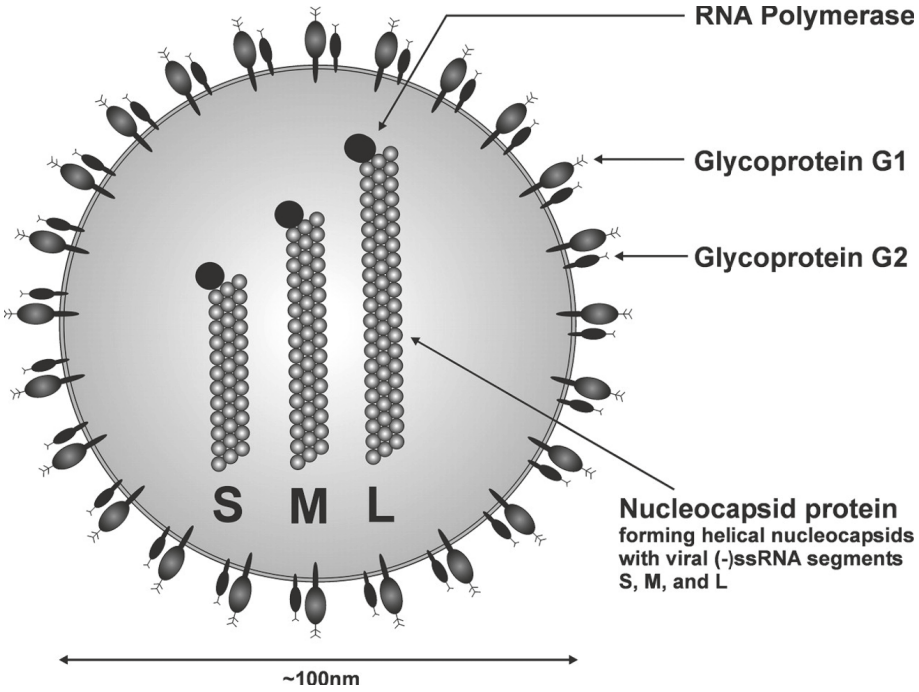
Figure 1. Regional distribution of clinically relevant hantaviruses in the world. (Figure adopted from Dethlev K, Figueiredo L et al. Hantaviruses - Globally emerging pathogens. J Clin Virology 2015:64:128-136. Copyright licence number 4904120224202)



The diameter of the hantaviruses ranges from 80 to 120 nm [21]. Different hantaviruses have similar trisegmented genomic organization. The small (S) segment encodes a nucleocapsid (N) protein which encapsulates the genomic RNA into three viral chromosomes. The large segment (L) encodes viral RNA-dependent RNA polymerase (L protein) responsible for the transcription and replication of the viral genome. The medium segment (M) is a precursor of two surface glycoproteins (G), i.e. Gn and Gc [24-26]. Hantavirus genome segments (RNA-dependent RNA polymerase) and N protein are stored within the round virus particle covered with spikes formed by Gn and Gc units as presented in Figure 2, adapted from a published article by Muranyi et al [27]. Differences in the pathogenicity could be related to the differences in the composition of these glycoproteins [28]. Genetic diversity, and the typing of different virus strains, is based mainly on point mutations and small insertions/deletions resulting in amino acid differences usually in the S and M genomic segments. In addition, reassortment of genome RNA segments occurs [25].

The main receptor for virus cell-entry belongs to the integrin family, according to *in vitro* studies with endothelial cell cultures. These β3-integrins also have an important role in regulating vascular integrity and endothelial cell permeability. Pathogenic hantaviruses mainly use $\alpha_v\beta_3$-integrin, which is also one of the main integrins in the podocytes, i.e. glomerular endothelial cells [28]. There is evidence that other molecules, most recently procadherin, also contribute to the cell entry of hantaviruses [29]. The virus can also use various, cell-type -specific endocytic pathways [30]. When the virus particles succeed in replicating in the Golgi apparatus, they exits the cell, probably via exocytosis, to infect new cells [24].

Figure 2. Schematic figure of hantavirus structure. (Adopted from Muranyi, Bahr et al. "Hantavirus infection" J Am Soc Nephrol 2005 16(12):3669-3679, picture reprinted with permission from the publisher)



2.1.2 Epidemiology of Puumala virus infection

PUUV infection is the most common hantaviral disease in Europe. The carrier rodent, the bank vole *Myodes glareolus*, is found all over Europe, excluding the Mediterranean regions, non-mainland Britain, most of the Spain and the northern tundra parts of Finland, Norway and Sweden [6].

Puumala virus was first isolated from the lungs of its reservoir host, bank vole, and named after the place where the animal was caught in 1977 [3]. Previous to that, a febrile illness called nephropathia epidemica, was described in Finland [4, 31].

The incidence of PUUV infection is known to fluctuate both geographically and in a timely manner in accordance with bank vole populations. In Finland, varying PUUV infection incidence rates can be explained by the number of predators and the possibility of the bank vole to hide and to seek shelter under the snow. After a favourable winter and advantageous summer, the bank vole populations can grow

fast. In such years, high incidence rates of PUUV cases are reported, typically with one to two peaks between August and January and with higher peaks in every three to four years [32]. In other northern European countries, infections parallel bank vole populations [5, 33]. In Sweden, the greatest reported outbreak was in 2007, when the incidence reached over 300 cases/100 000 persons in the Västerbotten region [34]. In central Europe, epidemics of PUUV infections follow years when the amount of oak-tree masts and other tree seeds favour rodents' reproduction, and the highest amounts of reported cases usually occur in late summer [35, 36]. The annual incidence in central Europe, for example in Germany, was 0.87/100 000 persons in 2001 to 2012 [37].

There can be large yearly variations in the bank vole populations and infections between different parts of Finland. At the time of the greatest level, in year 2008, over 3000 cases of serologically confirmed cases were recorded. The annual incidence in Finland is on average 31 cases/100 000 persons, with regional variation of 15/100 000 to 90/100 000 [5, 38, 39]. A high incidence has been reported in endemic areas such as Southern Savo, where in some area the seroprevalence can be as high as 55 % in men over 60 years of age [13, 38]. Seroprevalence of up to 12.5 % in the Finnish population are the highest reported in the world [40]. Seroprevalence studies in the northern parts of Sweden have shown increasing numbers, with a local highest seroprevalence of 13.4 % in 2009 [41].

2.1.3 Hantaviral disease caused by Puumala and other hantaviruses

PUUV causes a mild form of "hantavirus fever" in humans called hemorrhagic fever with renal syndrome (HFRS). This type of HFRS caused by PUUV is also known as nephropathia epidemica to separate it from HFRS caused by other hantaviruses. This clinical syndrome is recognized mainly in Europe, Western Russia and Asia. In Finland, PUUV is the only hantavirus known to cause HFRS. The other HFRS-causing hantaviruses in the Europe include Dobrava-Belgrade viruses (DOBVs) now divided into genotypes Dobrava, Sochi, Kukino and Saaremaa [42]. They are spread by mice in central Europe, the Balkan countries and Western Russia. Excluding mainly subclinical Saaremaa virus infections, DOBVs generally cause more severe HFRS compared with PUUV-related disease, with a fatality rate of up to 0.3-12 % [42]. HFRS in Asia is predominantly caused by Hantaan (HTNV) and Seoul (SEOV) viruses [21]. A few cases of SEOV infections, spread by rats, have also been reported

in Europe and other continents, making it the only hantavirus with worldwide distribution so far [43].

The most severe form of hantavirus fever, hantavirus cardiopulmonary syndrome (HCPS), also called hantavirus pulmonary syndrome (HPS), occurs in the Americas. The reported mean annual number of cases in the United States is around 30 [44]. In South America HCPS it varies from 50 to 75 cases per year, predominantly reported in Argentina, Chile and Brazil [2]. Typical viruses causing HCPS are Sin Nombre virus (SNV) in North America and ANDV spread in South America. HCPS is a severe disease with a fatality rate of around 35-40 % [21, 44]. The hallmark findings of this disease are thrombocytopenia, severe respiratory illness, non-cardiogenic pulmonary oedema with hypoxaemia and with increased capillary leakage [45, 46]. Renal insufficiency, i.e. elevated plasma creatinine level in HCPS has been detected in 40 % of the patients, but the prevalence of proteinuria, usually described as mild in HCPS, is not known [44].

Human exposure to hantavirus is considered to occur by way of aerosols, inhaling the dust containing animal excreta contaminated by the virus. The lungs may not be the only possible entry route, since the ANDV is capable of tolerating gastric juice at pH >3 and it infects intestinal cells *in vitro* as well [47]. PUUV antigen and viral RNA has been detected in the intestines of patients with acute PUUV infection, in endothelial cells of the capillaries in the lamina propria [48]. The virus can survive in an infectious form for two weeks at room temperature and even longer in colder temperatures. The virus prefers wet conditions and tolerates low temperatures from +4 to -20 °C, especially when not exposed to sun light [24, 49, 50].

In seroprevalence studies and patient cohorts, more infections have been detected in males than females. The male-to-female ratio in HFRS infections varies from 2:1 to 5:1 [13, 38, 51, 52]. Smokers are over-represented in populations with positive PUUV serology and smoking is associated with an increased risk of infection [40, 41, 53]. The infection induces protective immunity against re-infections, probably for a lifetime. Immunoglobulin (Ig) G antibodies against PUUV have been detected after 50 years [54].

2.2 Clinical presentation of Puumala virus infection

2.2.1 Symptoms and clinical findings

The clinical outcome of PUUV infection varies from asymptomatic or mild to fatal. The incubation time until the onset of symptoms varies from one to eight weeks [22]. According to the known seroprevalence in the Finnish population, and reported cases, serodiagnosed infections have been estimated to represent 13-30 % of all PUUV infections in the 90's [38, 55]. The vast majority have perhaps very mild symptoms or a subclinical infection. Severe life-threatening disease with shock, severe bleedings and pulmonary oedema, resulting in the need of intensive care unit (ICU) treatment is rare. Only 1-2 % of hospitalized patients experience hypotension with shock symptoms [52]. PUUV-related HFRS is characterized by a low fatality rate of only <0.1-0.4 % [12, 13, 15, 51, 56]. In 1995-2008, 13 deaths attributable to PUUV infection were observed in Finland [13].

Around half of the patients with serologically verified infection were hospitalized in 1995-2008 in Finland [13]. The great majority of patients have fever as the first symptom [15]. Of hospitalized patients, 35-65 % suffer from headache, backache and muscle and abdominal pains [52, 57, 58]. Gastrointestinal symptoms such as abdominal pain, diarrhoea and vomiting are reported by 30-50 % of the patients [11, 58]. As a pulmonary symptom, 10-50 % of the patients report dyspnoea or a dry cough [52, 59]. Transient blurred vision is a typical symptom reported by 30 % of the patients [60]. Thickening of the lens with myopic shift is seen in some patients with vision changes [61, 62].

Although considered as haemorrhagic fever, severe bleeding complications are rare, reported in less than 5 % of the patients. Gastrointestinal haemorrhages, epistaxis, macroscopic haematuria, conjunctival haemorrhages and petechiae are reported in 10-14 % of the patients [15, 58, 60]. In one study, haemorrhagic gastropathy was detected in all ten patients examined by gastroscopy 1-4 weeks after the onset of symptoms [63]. Panhypopituitarism due to hypophyseal haemorrhages and necrosis is a severe rare complication of PUUV infection, which can occur acutely with sudden vision loss. In some fatal cases with hypophyseal haemorrhage, PUUV N-protein has been detected in the hypophyseal cells and brain stem [64, 65].

During the clinical course of infection, five distinct phases have been identified: febrile, hypotensive and oliguric phases followed by polyuric and convalescent

phases. Not all phases are always present or obvious, especially in mild disease, and the time scales of different phases can vary [6, 18, 19, 66].

2.2.2 Laboratory findings

Three pathognomonic findings that characterize PUUV-induced HFRS at the acute phase are thrombocytopenia, signs of increased capillary leakage and acute kidney injury (AKI) with haematuria and proteinuria. Renal and urinary findings are discussed in detail below (Section 2.3). The incidence rates of typical laboratory findings according to four clinical studies, in Sweden [58], Finland [11], Germany [57] and France [60] are described in Table 2.

Thrombocytopenia (blood platelet count below 150×10^9/l) was detected in 90 % of 546 Finnish patients hospitalized because of acute PUUV infection [67]. It was associated with many disease-severity markers such as variables reflecting inflammation and capillary leakage, but not with the highest measured plasma creatinine concentration. In German patients, however, a low platelet count has been associated with disease severity and high plasma creatinine levels [68]. Also, in the study of 205 French patients, a level of platelets of $<90 \times 10^9$/l was predictive of severe disease including severe AKI [60]. In 47 patients with a documented platelet nadir, a low platelet count was associated with a higher plasma creatinine peak and the nadir occurred two days before the creatinine peak. The platelet nadir was reached at day five after the onset of symptoms and the platelet count was normal after the 10th day of illness in all patients [69]. In a Swedish study of 106 patients, the association between a low platelet count and more severe renal failure was not confirmed [70].

Several markers of the coagulation system show abnormalities during the acute PUUV infection, even without severe clinical coagulation problems. The criteria for disseminated intravascular coagulation are fulfilled in 18-28 % of the patients. [70, 71]. In fatal cases, a severe bleeding tendency is reported in substantial proportion of patients [15].

The degree of increased vascular leakage can be evaluated only indirectly by way of laboratory markers such as elevated haematocrit and haemoglobin levels, sometimes strikingly high as a result of haemoconcentration. This can be accompanied by clinical shock symptoms and pulmonary interstitial fluid accumulation with hypoxaemia in severe cases [72]. Hypoalbuminaemia can result

from capillary leakage but can also be explained by other causes such as acute inflammation and occasionally by nephrotic-range albuminuria.

Electrolyte changes are usual during the hospital stay. Plasma sodium concentrations are influenced by the fluid balance and can be decreased in the oliguric phase as a result of fluid accumulation, while they tend to rise in the polyuric phase because of water depletion related to excessively diluted urine. Of note, in connection with renal impairment, hyperkalemia is remarkably rare.

Inflammatory markers such as plasma leukocytes and C-reactive protein (CRP) are elevated in acute PUUV infection. The role of inflammatory markers is discussed in detail in Section 2.4.

Table 2. Incidence of typical laboratory findings in acute PUUV infection in four studies.

	Settergren (1) Sweden 1988 n=355	Mustonen (2) Finland 1994 n=126	Braun (3) Germany 2010 n= 75	Hentzien (4) France 2018 n=205
Elevated plasma creatinine	99 %	94 %	96 %	nr
Severe acute kidney injury [a]	33 % (serum creatinine >500 µmol/l)	nr	nr	20 % (plasma creatinine >353.6 µmol/l)
Hyponatremia	nr	58 %	nr	nr
Hypokalemia	nr	31 %	nr	nr
Hyperkalemia	nr	12 %	nr	nr
Anemia	nr	50 %	nr	nr
Thrombocytopenia	62 % <100 x 10^9/l (n=58)	75 %	69 %	49 % <90 x 10^9/l
Leukocytosis	nr	50 %	nr	23 %
Lymphopenia	nr	nr	59 %	nr
Elevated C-reactive protein	nr	nr	100 %	>100 mg/l in 35% of patients
Elevated liver enzymes	nr	34 %	nr	nr

Abbreviations: nr = not reported, AKI = acute kidney injury
[a] according to severe acute kidney injury definition used by the authors

1. Settergren B, Juto P, Trollfors B, Wadell S, Norrby SR. Hemorrhagic Complications and Other Clinical Findings in Nephropathia Epidemica in Sweden: A Study of 355 Serologically Verified Cases. J Inf Dis 1988;157(2):380-2
2. Mustonen J, Brummer-Korvenkontio M, Hedman K, Pasternack A, Pietilä K, Vaheri A. Nephropathia epidemica in Finland: a retrospective study of 126 cases. Scand J Infect Dis 1994; 26: 7–13
3. Braun N, Haap M, Overkamp D, Kimmel M, Alscher MD, Lehnert H, Haas CS. Characterization and outcome following Puumala virus infection: a retrospective analysis of 75 cases. Nephrol Dial Transplant 2010;25 (9):2997-3003
4. Hentzien M, Mestrallet S, Halin P, Pannet LA, Lebrun D, Dramé M, Bani-Sadr F, Galempoix JM, Strady C, Reynes JM, Penalba C, Servettaz A. Bioclinical Test to Predict Nephropathia Epidemica Severity at Hospital Admission. Emerg Infect Dis 2018 Jun;24(6):1045-1054.

2.2.3 Radiological and other findings

Abnormal chest X-ray findings are common in patients with acute PUUV infection. In a retrospective cohort of 380 PUUV patients, 35 % showed abnormal findings [73]. Pleural effusion, parenchymal infiltrates and atelectasis correlated with disease-severity markers, including high plasma creatinine levels. High-resolution computed

tomography (HRCT) showed lung parenchymal abnormalities in 12 out of 13 studied patients. Some patients also showed lymphadenopathy in the lung HRCT [74]. Impaired pulmonary diffusion capacity as a sign of alveocapillary lesions has been observed in over half of 13 analysed patients, mostly without clear pulmonary symptoms [75]. PUUV antigen has been detected in macrophages of the bronchoalveolar lavage (BAL) fluid [76], together with clear T-cell activation in the bronchial submucosal biopsy samples in patients with acute PUUV infection [77]. Pulmonary involvement in PUUV infection can sometimes result in severe respiratory insufficiency and pulmonary oedema, similar to that observed in HCPS [76].

In ultrasonographic examination the enlargement of the spleen has been seen in half of the patients and hepatomegaly in 13 % of cases [57]. In another study, splenomegaly was detected in magnetic resonance imaging in all 20 studied PUUV patients and the change in size compared with that in the recovery phase correlated with CRP levels, but not with thrombocytopenia or other disease- severity markers [78].

Several cardiac findings have been reported during the acute phase of PUUV infection. In a Finnish cohort of 70 patients, transient electrocardiogram (ECG) changes in serial examinations were found in 57 % of the patients. In serial echocardiographs of these 70 patients, left-ventricular contraction abnormalities were found in six patients and one patient had pericardial fluid [79]. ECG abnormalities were also detected in 18 %-50 % of the German patients with T-wave inversion as the most common ECG abnormality [37, 80]. Relative bradycardia, defined as a lower pulse rate than expected in the context of elevated body temperature, has been detected in 80 % of the patients [80].

Headache is the most common neurological symptom, but dizziness, confusion and sometimes convulsions have been reported in some (<10 %) patients [11]. Possible central nervous system (CNS) involvement was analysed in 58 hospitalized patients with brain magnetic resonance imaging (MRI), showing abnormalities in 29 % of cases, including pituitary abnormalities in six patients. Half of the patients had signs of CNS inflammation in cerebrospinal fluid, including positive PUUV IgM and elevated protein levels or leukocyte counts [81]. PUUV RNA has been found in cerebrospinal fluid explaining symptoms similar to those associated with aseptic meningitis [82].

2.3 Renal findings in Puumala virus infection

2.3.1 Acute kidney injury

Oliguric and sometimes anuric AKI with haematuria and proteinuria are the main renal findings in PUUV infection. Elevated plasma creatinine levels are detected in 88-95 % of hospitalized patients [12, 83]. The highest plasma creatinine values are typically observed 8-10 days after the beginning of symptoms i.e. fever [12, 37, 68]. A transient need for dialysis occurs in 3-6 % of patients [12, 56, 83].

In abdominal ultrasonography, often done because of abdominal and flank pains, the kidneys may appear to be swollen or show increased echogenicity [57, 84]. In a retrospective German study of 75 patients, renal ultrasonography revealed pathological changes in over half of the patients [57]. In a prospective Finnish study of 23 patients, an increase in renal length was seen in every patient during the acute phase of PUUV infection. Perirenal, pleural, and/or pericardial fluid accumulations were found in 13 patients. The severity of the findings was associated with the severity of AKI and fluid overload [84, 85]. Whether the pains originate from kidney swelling and associate with kidney capsule distension can only be suspected.

Renal recovery starts typically when polyuria (urine amount of over 3500-4000 ml/day) occurs, usually within two weeks after the onset of symptoms. Polyuria or diluted urine as a sign of a concentration defect can be suspected when plasma sodium concentration rises and body weight drops. The reported prevalence of polyuria varies from 45 %-87 % in hospital-treated patients [15, 57, 58, 86]. The median duration of polyuria is five days and the degree of polyuria has been associated with oliguria or anuria and severe AKI during hospital stay [86]. Notably, kidney function recovers within a few weeks and the recovery is usually complete in the majority of patients [12].

The incidence rates of urinary findings in acute PUUV infection according to four studies are presented in Table 3 [11, 57, 58, 60].

Table 3. Incidence of urinary findings in acute PUUV infection in four studies

	Settergren (1) Sweden 1988 n=355	Mustonen (2) Finland 1994 n=126	Braun (3) Germany 2010 n=75	Hentzien (4) France 2018 n=205
Haematuria	73 %	58 %	65 %	48 %
Albuminuria	89 %	83 %	82 %	81 %
Nephrotic-range albuminuria	nr	25 %	50 %	nr
Glucosuria	17 %	9 %	8 %	nr
Leukocyturia	nr	28 %	9 %	nr

Abbreviation: nr = not reported

2.3.2 Proteinuria

Proteinuria is typically an early sign detected soon after the onset of symptoms in PUUV infection. Elevated daily proteinuria of more than 200 mg can be found in over 80 % of patients. Nephrotic-range proteinuria (>3500 mg/d) is seen in a substantial proportion (25-50 %) of patients [11, 57, 87].

When renal tubular function is disturbed, lower-molecular-weight proteins such as α_1-microglobulin and β_2-microglobulin, which are freely filtered by the glomerulus, fail to be reabsorbed by the proximal tubule. In PUUV-infected patients the excess of these small proteins is found in the urine [88]. Proteinuria in PUUV infection can be defined as unselective, since besides these small proteins, urine contains mostly albumin and even larger proteins, like IgG, suggesting a glomerular origin [87].

α_1-Microglobulin is a small glycoprotein and a member of the lipocalin protein family, present in almost all tissues but synthesized mainly in the liver. The functions of α_1-microglobulin are not entirely clear, but it has immunosuppressive properties and serves as a radical scavenger, also described as a waste-bin for tissues. It is freely filtered in the glomeruli and reabsorbed by the proximal tubule cells where it is catabolized. Quantitative tissue-distribution studies have shown that α_1-microglobulin is most abundant in the liver, blood and kidney, in proximal tubular cells, and for example, in placenta where it might protect fetus from maternal immune system attack. Plasma concentrations of α_1-microglobulin are relatively stable, even in many inflammatory states. [89] Elevation of plasma concentrations is related to a decrease in glomerular filtration rate (GFR), but α_1-microglobulin is not regarded as a uremic toxin. Urinary α_1-microglobulin is considered to be a sensitive

indicator of impaired tubular function, and elevated urinary concentrations are observed in cases of both acute and chronic tubular damage [90, 91].

Reports about the disappearance rate of proteinuria soon after PUUV infection are few. The degree of albuminuria was clearly decreasing in 36 patients during hospital stay but it was still present in a few patients followed up to 13 days after fever onset [14]. In 34 patients with DOBV-induced HFRS, the amount of urinary glomerular proteins, albumin and IgG, on hospital admission were not associated with the need for hemodialysis during hospital stay. The urinary α_1-microglobulin/creatinine ratio on admission, however, was associated with a need of dialysis in four cases in this cohort. The amounts of urinary albumin and IgG were clearly decreasing in these patients at last control samples checked 10-17 days after admission [92]

Long-lasting proteinuria is a rare finding after PUUV infection. In cohorts with controls after 3-10 years, daily proteinuria and albuminuria levels were in the normal range, although slightly higher than in healthy controls. Overnight urinary excretion of α_1-microglobulin was elevated in nine out of 35 patients at five to six years after infection and had disappeared at ten years. The persistence of tubular proteinuria was not affected by the degree of AKI in the acute phase. [93, 94]

2.3.3 Haematuria

Microscopic haematuria has been reported in the majority (60-90 %) of patients in the early phase of PUUV infection. Haematuria can sometimes be macroscopic, in around 7 % of cases [11, 58]. In one study, the prevalence of microscopic haematuria was evaluated in a cohort of 70 consecutive patients with acute hospital-treated PUUV infection. Haematuria, defined as a positive urinary dipstick test result for erythrocytes and >2 erythrocytes/high-power field, was detected in 94 % of the patients [95]. Haematuria's ability to predict severe AKI was evaluated in 137 PUUV infected patients with normal kidney function at the time of diagnosis. In univariate model haematuria associated with severe AKI [83].

In a German study, haematuria was found in 74 % of NE patients on admission, and its presence was associated with the development of polyuria during hospital care [86]. Haematuria was present in 68 % of NE patients with severe thrombocytopenia and in 61 % of those with non-severe thrombocytopenia [69].

The prevalence of haematuria in AKI and the pathogenesis of haematuria in other kidney diseases are not well understood [96]. In 110 patients with biopsy-proven

ATIN of varying etiology and without glomerular changes, haematuria was present in 60 % of the patients and associated with higher proteinuria, need for renal replacement therapy and a higher relapse rate [97].

2.3.4 Glucosuria

The presence of glucosuria has been reported in a few earlier studies on PUUV infection, with the highest reported prevalence being 17 % in hospital-treated cases, without specifying if presented with elevated blood glucose or not [11, 57, 58]. In general, glucosuria is usually associated with high blood glucose. When reabsorption of the glucose from the primary urine by the proximal tubule is incomplete, the underlying reasons can be a tubular function defect, and hyperfiltration. The normal kidney threshold level for blood glucose is 10 to 11 mmol/l. Hyperfiltration-associated glucosuria, with a decrease in threshold level, can be seen in pregnancy as a physiological phenomenon. Glucosuria as a consequence of a proximal tubular function defect has been reported in human immunodeficiency virus (HIV) nephropathy associated Fanconi syndrome, regarded as possible side effect of tenofovir used in the treatment of HIV infection or the HIV nephropathy itself [98]. In pediatric patients, glucosuria is often detected in tubulointerstitial nephritis and uveitis syndrome [99]. In adults, glucosuria is infrequently reported in cases of ATIN of varying etiology [100]. The prevalence and significance of glucosuria in AKI is not known.

2.3.5 Renal biopsy findings

In human kidney biopsy samples, PUUV antigen in the tubular endothelium was first detected in 1996 by immunoperoxidase staining during the acute phase of infection [101]. Since then, hantaviral N protein has been found in tubular and glomerular cells in renal biopsy samples during acute PUUV infection [102]. The typical abnormalities reported in kidney histology are tubulointerstitial changes. Medullary haemorrhages in kidney biopsy samples have been found in 20-60 % of the patients, in some cases localized around hyperaemic peritubular capillaries. Interstitial inflammation and cell infiltrations are typically found [16, 103]. In a French study, kidney biopsy samples from 17 PUUV-infected patients with proteinuria (mean 2,5 g/day) were studied. The most common histological finding was ATN of varying degree in 15 patients. Other common findings included

medullary haemorrhages, microvascular inflammation, and cortical peritubular capillaritis with endothelial cells swollen and detached from the basement membranes. Interstitial inflammation was mild or moderate in most cases, and significantly milder when compared with controls with ATIN due to other reasons [17]. Glomerular changes are usually mild in light-microscopic studies. Slight mesangial hypercellularity is reported, but no clear glomerular injury. The amount of proteinuria has not been associated with any particular histological findings in light-microscopy [16, 17].

Immunohistochemical studies show that interstitial infiltrates consists of lymphocytes, plasma cells, monocytes/macrophages and leukocytes, mainly eosinophilic granulocytes and neutrophils [104]. Immunofluorescence studies of glomeruli in PUUV infection have mainly revealed negative for immunoglobulin (Ig)A, IgG, IgM, fibrinogen and complement components C1q and C3 [16, 17].

Electron microscopic (EM) studies of kidney biopsy samples have revealed alterations in the glomerular filtration barrier. Thickening of the glomerular basement membrane was described already in an EM study in the 70's, when about half of the 18 patients with acute PUUV infection underwent biopsies within two weeks after the onset of fever [105]. Slight segmental podocyte foot process fusion was also occasionally seen in early studies [103]. Recently, a case report showed clear podocyte foot process fusion in a PUUV-infected patient with rapidly reversible proteinuria in a kidney biopsy at seven days after the onset of fever [106]. An EM study showed podocyte foot process effacement in two out of three patients with PUUV infection in France, with no electron-dense deposits [17].

2.4 Pathogenesis and host response to Puumala virus infection

The symptoms of the infection are considered mainly to be consequences of the host immune response. When clear symptoms are present PUUV infection can be characterized as hyperinflammatory syndrome [107, 108]. Damage to the host's organs can occur as a result of disturbances in cellular processes caused by virus replication with or without cell death. The infection can result in changes in the sensitivity of the cells to soluble factors provoked by the host defense mechanisms [109].

Viral load could also affect disease severity, as supported by the results of *in vitro* studies of its effect on endothelial cells, and it could also explain differences in disease severity between mild and severe, in PUUV and DOBV induced HFRS

patients [110, 111]. In patients with PUUV infection, the association of higher plasma level of viral RNA with disease severity was not found [112].

Hantaviruses are capable of infecting many types of cells. The virus replicates mainly in the endothelium, but the cytotoxic T lymphocytes and mononuclear phagocytes as viral antigen presenting and immunomodulating cells, are considered important in the pathogenesis and severity of infection [107]. Endothelial function alterations are considered as the main causes for hantaviral disease manifestations in many different organs and all of the vasculature.

Understanding and investigation of the pathogenesis of hantaviral disease have been impeded by the lack of adequate animal models. As asymptomatic carrier animals, rodent models are of limited value. High amounts of neutralizing antibodies and responses favouring upregulated anti-inflammatory agents in carrier hosts animals could explain their chronic infection without symptoms [24]. Investigations of PUUV infection in macaques have shown similarities in the cytokine responses, urinary findings and histopathological changes in comparison with humans and, as analogous to humans, a high proportion of asymptomatic infections [113].

2.4.1 Endothelial dysfunction

Endothelial cells are the main sites for PUUV replication, but substantial endothelial cell damage is not seen histologically. Many signs of altered endothelial function in PUUV infection can reflect capillary leakage [10, 18]. *In vitro*, there are no clear endothelial activation in cells infected by PUUV, but the activation can be induced with sera from infected patients [114]. A host inflammatory response is thus considered essential for the endothelial dysfunction.

Hantavirus infection results in a change in the expression of human leukocyte antigen (HLA) molecules in endothelial cells. Increased expression of certain HLA molecules enables a change in the interaction of a variety of immune cells such as CD8+ T cells, natural killer (NK) cells and neutrophils, whose cytotoxic activity probably contributes to vascular leakage [107]. Platelets also have a role in maintaining vascular integrity and complex disturbances in this homeostasis with endothelial cells have been documented in a variety of viral hemorrhagic fevers, although platelet-virus interaction is poorly understood when it comes to hantavirus infections [115].

Soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular adhesion molecule 1 (sVCAM-1) are endothelial cell-contact molecules. Although

PUUV-infected endothelial cells express elevated levels of sICAM-1 *in vitro* [114] and elevated levels of ICAM-1 have been detected in the kidneys in cases of PUUV infection [104], as well as in blood samples during acute infection [116] any correlation between sICAM-1 and clinical kidney injury markers has not been proven. Vascular integrity is maintained also by angiopoietins which have showed signs of dysregulation in patients with PUUV infection [116, 117].

Vascular endothelial growth factor (VEGF) protein family include signal proteins involved in the alteration of permeability in micro vessels. The permeabilizing effects of VEGF in angiogenic endothelium is mediated by β3-integrins. Levels of VEGF is proved to be elevated in both hantavirus diseases, HCPS and HFRS. [109]. In one study, when daily alterations of VEGF concentrations in the plasma and urine were evaluated during DOBV and PUUV infection, no clear association with disease severity was found [118]. VEGF can also be associated with endothelial repair, since in HFRS, concentrations remain elevated in the convalescent phase [116].

2.4.1.1 Podocyte dysfunction

Podocytes are special types of endothelial cells in the kidney glomeruli and a crucial part of the filtration barrier. Changes in the multiprotein complex of podocyte-specific variants of adherence proteins and tight junctions between cells, making up the slit diaphragm, form a selective paracellular route of solute flow and also a barrier against larger proteins. Hantavirus infection *in vitro* results in changes of distribution and decreased expression of ZO-1, a marker of endothelial tight junctions and slit diaphragm in podocytes and renal tubular endothelial cells [102]. This phenomenon was also observed in kidney biopsy samples from seven PUUV infected patients. The reduced intensity of ZO-1 staining in these patients was correlated to reduced serum albumin as a possible link to albuminuria, although this was not discussed by the authors [102].

Hantaviral effects in the glomerular cells are supported by the results of *in vitro* studies. Hantavirus replication in human tubular endothelial cells and podocytes and the subsequent expression of N protein in these cells have been associated with alterations in cell structure and migration functions. Neither supernatants derived from infected podocytes nor sera from patients with hantavirus infection had this effect *in vitro* [119].

2.4.2 Immune responses

2.4.2.1 Cytokine response

The levels of several plasma cytokines are elevated in the early phase of PUUV infection [120, 121]. Compared with healthy controls, the main proinflammatory cytokines interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1beta and IL-1 receptor antagonist (IL-1Ra) were clearly elevated in plasma early in the course of the disease in 70 patients with PUUV infection. The most marked elevation was observed in plasma IL-6 level. Plasma or urinary excretion of IL-6 did not correlate with plasma creatinine level. Urinary excretion of IL-6 correlated however with albuminuria, proteinuria and urinary excretion of IgG, while plasma IL-6 level did not. The amounts of plasma and urinary excretion of IL-6 were already decreasing during the first three hospital days, suggesting that the peak excretion of IL-6 exists even earlier. [95]

In a PUUV-infected cohort of 36 patients, with severe AKI in 15 cases, high amounts of IL-8 and IL-6 were found in the plasma and urine [14]. Although plasma IL-8 concentrations were not correlated with higher plasma creatinine values, serial urinary cytokine measurements showed that both maximum level of both urinary IL-6 and IL-8 correlated with AKI severity [14]. In a larger cohort, plasma IL-6 levels correlated only slightly with plasma creatinine values but more clearly with proteinuria, thrombocytopenia, blood leukocyte count and many other disease-severity markers, such as duration of hospital stay, reduced minimum systolic blood pressure, and change in weight [108]. The better correlation of urinary vs. plasma IL-6 and IL-8 with AKI severity suggests that the amount of local cytokine production in the kidneys could affect the severity of AKI.

The type-2 cytokine transcription factor (GATA-3) is considered as a necessary transcription factor for innate type 2 T-cell response. Serial cellular RNA study from the urinary sediment in PUUV infection showed that the amount of GATA-3 was higher in the patients with severe AKI (defined as three-fold rise of creatinine) and the expression was highest at the first hospital days before highest creatinine values [14]. GATA-3 is expressed in the urothelial cells of collecting ducts in the adult kidney but the source of the urinary GATA-3 in PUUV infection is not identified. As certain cytokines have also antiviral properties, the balance of several cytokines probably affects the outcome. When cytokine responses were investigated throughout the course of the disease, the levels of several cytokines began to decrease before 20 days had elapsed [110].

2.4.2.2 T-cells

Antigen presenting cells such as monocytes, macrophages and dendritic cells produce interferons and also activate CD8+ T lymphocytes (cytotoxic T lymphocytes, CTLs, also called effector CD8+T cells) when presenting viral antigens. Vigorous cytotoxic T-cell responses, especially a cytotoxic CD8+ T cell population increase, is seen in peripheral blood at the early phase of the PUUV infection. The T-cell response peaks at two weeks after the onset of symptoms and decreases in parallel with viral load after a few weeks [122].

Neutrophil infiltration seen in kidney biopsy samples consists mainly of $CD8^+$ T cells [16]. Large numbers of $CD8^+$ T cells are found in endobronchial biopsy samples and in BAL fluid during the acute phase of the infection [77]. This $CD8^+$ cytotoxic lymphocyte response in the lungs associates with systemic disease-severity markers, including maximum creatinine values [123].

The cytopathic effect of this effector $CD8^+$ T-cell population towards infected endothelial cells is not seen in HFRS or HCPS. The mechanisms of cell-damage prevention are mainly unclear. Damage prevention can be partly explained by the ability of the virus to block cell cytotoxicity or inhibit apoptosis [124, 125]. Although lacking cytotoxicity, T-cells release cytokines that can result in endothelial dysfunction. After infection, PUUV-specific CD8+ memory T-cells have been detected for up to 15 years and viral N protein seems to be the main target of these cells when activated by PUUV *in vitro* [126].

2.4.2.3 Mononuclear phagocytes

In healthy adults, mononuclear phagocytes such as monocytes, macrophages and dendritic cells with migratory capacity reside in barrier tissues such as the mucosa of the lung and intestine, and lymph nodes. They are capable of initiating and regulating virus-specific immune responses. Mononuclear-derived cells and dendritic cells in the lungs are in close contact with respiratory endothelium where the infection takes place [127].

PUUV antigen has been detected in macrophages in tissue samples from fatal cases [128] and in macrophages in BAL fluid during acute PUUV infection with severe pulmonary insufficiency [76]. Dendritic cells are potent T-cell stimulators. There is an association between elevated levels of CD8+ T cells and mononuclear phagocytes in endobronchial biopsy samples [129]. The observed decrease in the number of mononuclear phagocytes in the peripheral blood in parallel with viremia

could be explained by their redistribution into tissues, including the airways [129]. It has been suggested that infected dendritic cells could protect virions against rapid inactivation by the innate immune system and spread the virus to endothelial cells during their transendothelial migration to extrapulmonary tissues [130, 131].

2.4.2.4 Natural killer cells

Natural killer (NK) cells are cytotoxic lymphocytes that have same the progenitors as T- and B-lymphocytes. They are activated by cytokines and are critical to innate immune system mediating antiviral responses [132].

PUUV-infected endothelial cells have been found to activate NK cells *in vitro* but they were resistant to NK-cell-induced lysis, while uninfected endothelial cells were killed. Virus replication was not observed in NK cells [133]. NK cells in PUUV-infected patients are found in low numbers at the early phase of symptom onset and they rapidly expand before the T-cell response and they persists for weeks after symptom onset [134]. The possible protective role of NK cells and their pathogenic significance in PUUV infection are not fully understood as there are no data about their correlation to the clinical severity of the infection.

2.4.2.5 Neutrophils

The role of neutrophils in viral infections is not clearly understood. An elevated level of peripheral blood leukocytes is recognized as a risk-factor of severe AKI in PUUV infection [14, 112]. The PUUV itself does not seem to activate neutrophils *in vitro*. However, endothelial cells infected by PUUV release factors that do so. As a strong chemotactic factor for neutrophils, IL-8 could play a major role in recruiting these cells. In kidney biopsy samples taken during acute PUUV infection, IL-8 was localized in the tubulointerstitial space and tubular epithelium where neutrophil infiltrates and their activation products were also seen. Neutrophil activation markers also correlated with the severity of kidney injury in these patients [114].

As a central cell type in the innate immunity response, neutrophils eliminate bacteria by phagocytosis and by releasing soluble anti-microbials agents by degranulation. They can also undergo a novel form of apoptosis called NETosis which results in the release of neutrophil extracellular traps (NETs) in both, bacterial and viral infections. NETs are net-like structures of double-stranded DNA coated with histones and antimicrobial molecules [135]. Circulating IL-8 levels have been

found to correlate with those of histones and other antimicrobial molecules released at degranulation of activated neutrophils in PUUV patients, suggesting that activation of neutrophils does occur in PUUV infection [114]. Double stranded DNA/histone complexes in the sera of HFRS patients have been found to be elevated in the acute and convalescent phase, at levels higher than in patients at the recovery phase of sepsis or influenza. NETs were also detected by immunofluorescence in three out of four kidney biopsy samples of hantavirus- (PUUV- and DOBV-) infected patients [136].

2.4.2.6 Humoral response

Immunoglobulins secreted by B lymphocytes in response to viral stimulus in PUUV infection are directed mainly towards viral N- and G-protein [137, 138]. IgM antibodies are present early and used in diagnostics [139]. Studies on HCPS patients suggest that low IgG antibody titers are associated with more severe disease, while associations concerning IgA and IgM responses are unclear [140]. Lower IgG responses in the early phase of the PUUV infection were associated with more severe disease, but no association was found with IgA or IgM responses, viral load or the longevity of viraemia [112]. Also, when PUUV-caused mild HFRS was compared with DOBV-induced more severe disease, the IgG response was slower in the severe form of HFRS [110]. Neutralizing antibodies develop in humans early in the disease course. It has been reported that HCPS patients with higher numbers of neutralizing antibodies have milder disease, but in HFRS, especially in PUUV infection, the role of neutralizing antibodies is unclear [110, 141]. Virus-specific IgG antibodies can stay detectable for decades after the infection [142].

Circulating immune complexes have been detected in PUUV patients, but there was a delay in the rise of the level and relatively long persistence of up to eight months with no correlation with clinical course [143]. A temporary presence of anti-DNA antibodies and anti-nuclear antibodies has also been detected in the acute phase of hantaviral infection [136].

2.4.2.7 Other markers of inflammatory response

Complement activation in acute PUUV infection can be detected by elevated levels of a complement end-product, membrane attack complex (SC5b-9) and a decrease of plasma C3 levels. These signs of activation in 61 patients correlated positively with

the length of hospitalization, change in body weight and with leukocyte count as disease-severity markers but only slightly with the rise in plasma creatinine level. The level of SC5b-9 correlated positively with abnormalities seen in chest X-rays [144]. Post-mortem studies of four fatal cases showed clear signs of complement activation especially in the lungs [65].

Plasma levels of a member of the pentraxin protein family, C-reactive protein (CRP) are elevated in acute PUUV infection but its associations with disease severity vary in different cohorts. In a German study, 137 patients with normal serum creatinine levels at the time of diagnosis were selected in order to identify factors predicting the severity of AKI. In this cohort, 12-fold increase in plasma CRP level was found as a risk-factor for severe AKI [83]. In Swedish patients, maximum CRP levels during hospital stay did not show a significant difference in mild vs. severe disease [112]. In a Finnish cohort of 118 patients, 29 % of them had CRP levels over 100mg/l, but no association between higher CRP levels and AKI severity was found. In logistic regression analyses, a high plasma CRP level was even found to be a possible protective factor as regards high plasma creatinine concentrations [108].

Another member of the pentraxin protein family, pentraxin-3 (PTX3) is synthetized mainly in the inflammation site by many cell types including mononuclear phagocytes and endothelial cells. The role of PTX3 in the immune response includes a capacity to bind complement C1q, activation of the classical complement pathway and interaction with complement regulators to prevent exaggerated activation [145]. PTX3 levels were elevated during acute PUUV infection studied in 102 patients and the levels were associated with higher plasma creatinine levels, and plasma IL-6 concentrations as well as other markers of disease severity [146].

The cell-membrane protein urokinase-type plasminogen activator receptor (uPAR) is a mediator of the immune system promoting the migration and adhesion of leukocytes. The plasma concentration of its' soluble form (suPAR) released by endothelial cells is considered to reflect inflammation. The roles of suPAR in kidney diseases have remained unclear although the results of *in vitro* and animal studies have suggested a role in the pathogenesis of proteinuria in FSGS and actions mediated by its high affinity to $\alpha_v\beta_3$ integrin [147]. In acute PUUV infection plasma suPAR concentrations correspond with the levels reported in bacteremic patients and correlate with AKI severity in a cohort of 97 patients [148]. Fractional excretion of urinary suPAR has been reported to be elevated in acute PUUV infection and correlated with maximum plasma creatinine and albuminuria. Plasma and urinary suPAR levels did not correlate contrary to reports concerning other inflammatory

diseases where plasma and urinary suPAR levels are usually well correlated [149]. Local production of suPAR in the kidneys when there is an on-going inflammatory process cannot be excluded.

One of the most studied new biomarkers of AKI is neutrophil gelatinase-associated lipocalin (NGAL) secreted by neutrophils and considered to be a tubular injury marker. Urinary levels of NGAL on hospital admission were associated with the severity of AKI in 61 patients with acute PUUV infection [150].

An inflammatory glycoprotein YKL-40, also known as chitinase 3-like protein is studied as a urinary biomarker of AKI [151]. Plasma levels YKL-40 were studied in 79 patients with acute PUUV infection. YKL-40 levels were elevated during acute infection compared to 15 days and 1year control samples and correlated positively with plasma creatinine, IL-6 and resistin levels [152]. The role of YKL-40 in AKI is not known, but a study in kidney transplant patients suggested that urinary YKL-40 could be a sign of tubular repair process associated with favorable outcome [153].

2.4.2.8 Adipokines

Adipokines such as adiponectin, leptin and resistin are molecules first found in adipose tissue and shown to have active endocrine functions. Since then, adipokines have proved to be secreted by many other cells too. The effect of adipose tissue on immune-system processes and chronic low-grade inflammation mediated by adipokines have shown that obesity is related to changes favoring proinflammatory adipokines [154]. In acute inflammation triggered by bacterial lipopolysaccharide endotoxemia in healthy volunteers, there were only slight changes in plasma leptin levels and no significant change in plasma adiponectin levels, but a rapid sharp rise of plasma resistin levels was observed, reflecting its role in acute innate immunity cascade [155]. The roles of adipokines in acute infections and AKI are not clear.

Adiponectin knockout mice have been found to be better protected against ischaemia/reperfusion-induced AKI, but the mechanism of action and the effects of adiponectin in human AKI are not known [156]. Leptin affects eating behaviour as well as the regulation of immunity [157]. Plasma leptin elevation has been detected in uremic hemodialysis patients, but not in AKI patients [158].

Resistin is considered to be a pro-inflammatory adipokine produced mainly by macrophages and neutrophils. It has the ability to activate endothelial cells, mediated by a toll-like receptor signaling pathway [159, 160]. Plasma resistin levels are less than 20 ng/ml in healthy subjects. It is regarded as uremic toxin and end-stage renal disease patients' plasma levels are reported to be around 30-40 ng/ml [161].

Resistin production is upregulated in sepsis reaching plasma levels of 40-70ng/ml and even higher than 100 ng/ml in septic shock and in septic shock with AKI [162, 163]. In emergency-department patients who met the criteria for sepsis, the elevation of plasma resistin levels has been found to associate with the severity of illness, namely septic shock, but not with mortality [162]. Resistin had a better discriminatory ability between sepsis and trauma-related systemic inflammatory response syndrome compared with CRP or lactate in 46 ICU patients. Patients with sepsis had slightly higher median creatinine concentrations and significantly higher resistin levels, but no significant difference in overall illness severity as defined by the Sequential Organ Failure Assessment score. The staging of AKI was not specified in this study [164]. Higher resistin levels in the plasma of AKI patients with sepsis resulted in similar impaired neutrophil migration capacity as recombinant resistin *in vitro*, suggesting that resistin could be the mediator of this dysfunction predisposing AKI patients to infections [163, 165].

Investigations concerning resistin or other adipokines in acute viral infections are scarce. There are no previous studies on resistin in PUUV-infected patients. In Crimean-Congo haemorrhagic fever, high plasma resistin levels were associated with a bleeding tendency and a low platelet count [166]. Plasma resistin concentrations have been found to be elevated in patients with non-severe acute Dengue fever, compared with healthy controls, in the early febrile phase of the infection. No association with platelet or leukocyte count was found while other clinical markers and other sequelae of the infection were not analysed [167].

2.4.3 The effect of host genetics

The significance of the degree and type of host immune response is supported by the discovery of genetic propensity for severe disease. HLA-B8-DR3 haplotype have been associated with severe kidney injury and shock symptoms in PUUV infection, while allele HLA-B27 positivity has been associated with a benign course of the disease [168, 169]. Relatively rare allele 2 of cytokine TNF-α correlated with an enhanced producer phenotype associated also with severe disease but maybe in conjunction with more common HLA risk allele and not as an independent factor [170, 171].

2.4.4 The effect of gender

Hantavirus infections show a male dominance. The male-to-female ratio in recognized HFRS cases varies from 2:1 in PUUV infections to 5:1 reported in infections caused by six different hantaviruses in Russia [13, 38, 51]. While the incidence of diagnosed symptomatic PUUV infections shows clear male dominance, this could indicate that males have more severe disease and females more subclinical infections. Behavioural and occupational factors affecting virus exposure and explaining male dominance cannot be excluded. In a Finnish study, seroprevalence in a healthy female population showed a similar proportion of infections as in a cohort of diagnosed symptomatic female cases per head of population in the same area, suggesting that there is no gender-related difference in symptomatic infections [38]. Both Finnish and Swedish seroprevalence studies of PUUV antibodies show higher numbers in men vs. women [41]. Sex-dependent differences in the cytokine responses have been detected in hospitalized patients, but the associations of these differences with clinical severity markers were not addressed [172]. In a German population, the severity of the disease did not seem to be affected by gender. The only difference detected was that males had ECG abnormalities more often than females [37].

2.5 Diagnosis

Antibody testing is the main method used in diagnostics of PUUV infection [173]. Serodiagnostic methods are based on detection of PUUV virus N-protein specific IgM or IgG [174] and the methods include enzyme immunoassays, immunoblotting and immunofluorescence assays. PUUV-specific IgM antibodies are used in the rapid immunochromatographic point-of-care tests now available [175]. IgM antibodies hardly never stay undetected after the 5th day of illness [176].

It is possible to detect viral RNA in peripheral blood by polymerase chain reaction (PCR) during the early phase of PUUV infection. The extent of viremia varies largely and depends on hantavirus type. Due to low level of viremia, such tests can be negative in a substantial proportion of patients [177]. Viral RNA in the circulation can remain detectable three weeks after disease onset, but PCR-tests can also become negative in the early phase of the infection and thus not suitable solely for reliable diagnostics [112].

2.6 Treatment

No specific treatment of PUUV infection exists so far. The treatment of patients is supportive, including pain control, maintenance of fluid and electrolyte balance as well as starting temporary dialysis in cases of severe and prolonged kidney failure. The aim is to achieve an optimal intravascular volume in order to maintain effective blood volume adequate to stabilize haemodynamics and to avoid fluid overload, which in association with capillary leakage could lead to pulmonary oedema.

As a treatment option for hantaviral diseases, antiviral drugs such as ribavirin in humans [178, 179] and favipiravin in animal models [180] have been tested, but without efficacy. Since a strong virus-specific IgG response is associated with a milder disease outcome, neutralizing monoclonal antibodies generated from ANDV survivors have been tested in animal studies, resulting in better survival of hamsters [141].

Increased capillary leakage with pulmonary oedema and shock symptoms in severe cases resistant to conventional supportive treatment in ICU, led to off-label use of a bradykinin receptor antagonist, icatibant, in patients with potentially lethal disease. This drug is used in hereditary angioedema in cases of serious swelling typical of that disease, mediated by bradykinin. Two case reports showing benefit have been published [181, 182], but the optimal timing, dose and administration route as well as real efficacy remains to be determined. As a non-immunological reaction, activation of the plasma kallikrein-kinin system, resulting in increased liberation of bradykinin and a change in cell permeability has been demonstrated in *in vitro* studies of hantavirus-infected (HTNV and ANDV) endothelial cells [183].

2.7 Long-term consequences

There are reports of hypertension associated with prior PUUV or other hantavirus infections in small cohorts and seroprevalence studies [4, 184, 185]. Although recovery of the kidney function is mainly complete, some patients have shown hyperfiltration 3-7 years after the infection, and also higher ambulatory blood pressure values after a few years compared to healthy controls (17 % vs. 3 %) [186]. The tendency was not associated with the disease severity at the acute phase or the HLA genotype associated with more severe acute disease [94].

Several case-reports have described an appearance of glomerulonephritis within a few weeks after acute PUUV infection in the convalescent phase. Most of the cases

expressed nephrotic-range proteinuria and haematuria with impaired kidney function. Membranoproliferative glomerulonephritis with both complement and immunoglobulins in the IF study of glomeruli was the most common finding in kidney biopsy sample. Membranous nephropathy has also been reported. The renal sequelae were mainly favorable as most patients achieved remission of nephrotic syndrome within median of half a year [187, 188].

In a Swedish retrospective registry data analysis, there was an increased risk of venous thromboembolic events after HFRS. The risk was highest within 2-4 weeks after acute infection and was higher in females than males. The incidence of thrombotic events within a year after infection seemed much higher than in reports about the incidence after other types of infections [189]. Swedish registry data have also shown that patients with recent PUUV infection may have an increased risk of acute myocardial infarction and stroke within a few weeks after infection [190]. Studies of acute PUUV infection have shown levels of plasma creatinine kinase, troponine I and T mainly within normal ranges [79, 80].

In the recovery phase of the PUUV infection, post-infectious fatigue is frequently complained by the patients. The incidence and duration of these symptoms was analyzed retrospectively by health questionnaires in 1100 Swedish patients. Over one third of the patients reported duration of symptoms over 3-6 months to full recovery [191]. The mechanisms underlying fatigue symptoms are mainly unclear but defects in hormonal systems are common during acute infection. Gonadal and/or thyroid axis abnormalities was detected in 56 % of 54 patients in the acute phase and 17 % of them developed a chronic defect within 5 years [192]. Hypopituitarism after PUUV infection occurring slowly showed signs of chronic hypophysitis suggesting autoimmune etiology [193].

In the analysis of Swedish registry data of over 6000 patients with verified PUUV infection, followed up by linkage to Swedish Cancer Registry, revealed a 73 % increased risk of developing lymphoma compared with general population. The risk was highest early, within a year, after acute infection. [194]

3 AIMS OF THE STUDY

The aims of the present study were:

1. To assess whether the amount of albuminuria or other urinary proteins has an association with the severity of kidney injury in acute PUUV infection. (I)

2. To evaluate if the degree of haematuria is associated with the severity of acute kidney injury and if the amount is associated with abnormalities of the coagulation system. (II)

3. To study whether glucosuria has an association with disease severity in PUUV infection. (III)

4. To assess the disappearance rate of albuminuria soon after acute PUUV infection, as well as to study whether the disappearance rate varies between patients. (IV)

5. To evaluate the alterations of plasma adipokine concentrations in acute PUUV infection and their associations with disease severity and other inflammation markers. (V)

4 PATIENTS AND METHODS

4.1 Patients

Since 1997, all consecutive patients admitted to the renal ward of Tampere University Hospital due to serologically confirmed acute PUUV infection have been asked to participate in the prevailing ongoing PUUV infection study. The patients were recruited as soon as possible but at the latest on the first working day after admission to hospital. All participating patients provided a written informed consent. The Ethics Committee of Tampere University Hospital approved the Study protocols (codes 97166, 99256, R04180, R09206) and the Study was conducted in accordance with the principles of the Declaration of Helsinki.

Patients included in Studies I-V are listed in Table 4 according to the time periods of hospital treatment. Altogether, 236 patients participated in the studies during these years. As presented in the Table 4, Studies I and II included the same patients and partly the same patients were also included in the Studies III-V. In Studies I and II, 205 patients were selected from 236 patients and the selection was based on the availability of at least two plasma creatinine values and a urinary dipstick test result on admission. Study III included 195 patients with at least one urinary dipstick test result for glucose on admission. In Study IV, all included patients underwent timed urine collection to assess the amount of albuminuria during the acute and/or convalescent phase. Study V included 79 out of 87 consecutive patients with plasma samples available for adipokine measurements.

Table 4. Numbers of patients in the Studies I-V according to time-periods of hospital treatment due to Puumala virus infection.

Hospital treatment time-periods	9/1997-12/1999	1/2000-12/2004	1/2005-12/2010	1/2011-12/2014	Of total number of 236 patients
Studies I and II	68	55	53	29	205
Study III	69	44	56	26	195
Study IV		59	55	27	141
Study V			55	24	79

The gender and age distributions of the patients included are shown in Table 5. The numbers of patients with prior diagnosis/diagnoses before acute PUUV infection in Studies I-II, III, IV and V were 56 (27 %), 59 (30 %), 52 (37 %) and 24 (30 %), respectively. The following diagnoses (and the number of cases in the studies, respectively) were hypertension (n=17/13/16/7), coronary artery disease (n=7/6/5/2), rheumatoid arthritis (n=5/4/4/3), diabetes type I or II (n=4/2/5/3), bronchial asthma (n=4/6/6/4), prostate hyperplasia (0/2/0/0), atrial fibrillation (n=0/0/4/0), hypothyroidism (n=4/0/0/0), coeliac disease (n=3/3/1/0), inflammatory bowel disease (n=2/1/2/0), epilepsy (n=2/1/1/0), gastritis/reflux disease (n=0/0/0/4), history of cerebral ischaemic attack (n=1/1/1/1), sequelae of renal tuberculosis (n=1/1/0/0), cardiac conduction disorder (n=2/0/0/0), psychiatric disorder (n=2/0/0/0), polycystic kidney disease (n=1/0/0/0), chronic lymphatic leukaemia in remission (n=1/0/0/0), spherocytosis (n=0/1/0/0), history of sarcoidosis (n=0/0/1/0), cardiac valve disease (n=2/0/0/0) history of operated malignancy (n=1/0/1/0), polyneuropathy (n=0/0/1/0), history of splenectomy (n=0/0/1/0), sleep apnea (n=0/0/1/0) and multiple sclerosis (n=0/0/1/0). None of the patients had a prior diagnosis of chronic kidney disease (CKD).

Table 5. Gender and age distributions of patients included in Studies I-V.

	Study I & II	Study III	Study IV	Study V
Patients (number)	205	195	141	79
Male (%)	67	68	68	61
Age, years median (range)	41 (15-77)	41 (21-73)	42 (21-73)	41 (21-74)

4.2 Methods

4.2.1 Study designs

All patients were physically examined and a detailed past and current medical history was recorded in the acute phase of PUUV infection. Clinical data and laboratory test results during hospitalization were recorded.

In Studies I-III and V, a urine sample for dipstick testing was obtained in all cases on hospital admission; already at the emergency room for clinical reasons. In Studies I and II, timed urine collection started on the first evening of hospital care in 70 consecutive patients hospitalized during 1997-1999. In those patients, samples for timed 24-hour urinary protein excretion as well as timed overnight urinary excretion of albumin, IgG and α_1-microglobulin were collected during the first three days at hospital. To assess the changes in these urinary findings in relation to daily plasma creatinine values, the delay between symptom onset and analysis was recorded.

In Study II, besides a urine dipstick sample on admission, a urine sample for sediment analysis was obtained in 189 cases. To evaluate possible factors affecting the degree of haematuria, levels of plasma fibrinogen, D-dimer and prothrombin fragments were also determined in 43 cases.

In Study III, glucosuria was assessed from the first urine dipstick test. A control urine dipstick test during hospitalization was obtained in 87 (40 %) of the 195 patients during hospital care.

In Study IV, serial measurements of overnight urinary excretion of albumin (cU-Alb) at different time-points of the disease were made to assess the disappearance rate of albuminuria soon after acute PUUV infection.

In Study V, plasma samples for the measurement of the adipokines resistin, leptin and adiponectin, as well as measurement of plasma CRP and IL-6, were collected once a day between 7:30-8:30, at a median of two (1-5) times during the hospitalization and the delay between symptom onset and sampling was recorded. Follow-up samples were obtained at control visits at a median of 15 (range 7-21) days after hospital discharge and at one year.

4.2.2 Diagnosis of Puumala virus infection

Patients were recruited by way of clinical suspicion of acute PUUV infection. The infection was confirmed by way of a single serum sample, by detection of the typical granular staining pattern in the immunofluorescence assay [138] and/or low avidity of IgG antibodies to PUUV [174] and/or PUUV IgM antibodies using an "in-house" enzyme-linked immunosorbent assay (ELISA) based on a recombinant antigen [195].

4.2.3 Clinical variables

The time of the onset of fever was recorded. The difference between minimum and maximum body weight during the hospital stay (to obtain body weight change during hospitalization) was used to reflect the disturbances in the fluid balance. First blood pressure values at the emergency-room and also minimum and maximum systolic and diastolic blood-pressure values during hospital stay were recorded. A systolic blood-pressure value under 90 mmHg, with shock symptoms, was designated as clinical shock. Daily urinary output recordings were taken from the patient records. As a clinical marker, the length of hospital stay was taken to reflect the overall severity of illness. The result of a chest radiography analysed by a radiologist was recorded in 150 patients (77 %) in Study III. Body mass index (BMI) was calculated using the formula kg/m^2, where kg is a person's weight in kilograms and m^2 is person's height in meters squared.

4.2.4 Basic laboratory blood tests

Plasma creatinine concentrations were obtained according to clinical indication. The highest plasma creatinine value measured during hospitalization was designated as

maximum. Plasma creatinine concentrations were determined by using Vitros assays (Johnson & Johnson, Rochester, NY) up to 1999, and by Cobas Integra assays (F. Hoffmann-La Roche Ltd., Basel, Switzerland) thereafter. Severe AKI (stage 3) was defined as a maximum plasma creatinine value ≥ 353.6 μmol/l according to KDIGO Guidelines [196].

Basic laboratory tests, such as those for blood leukocytes and platelets, haematocrit, electrolytes, plasma CRP, urea, glucose and albumin were carried out according to clinical indication and analysed by routine automated chemistry analyzers at the Laboratory Centre of the Pirkanmaa Hospital District (later named Fimlab Laboratories). The highest or the lowest values of each variable, were designated as maximum or minimum, as appropriate.

In Study II, plasma fibrinogen, fibrin degradation product D-dimer and prothrombin fragments F1+2 indicating thrombin formation were analysed in 43 patients during the acute phase. Plasma fibrinogen levels were assessed from citrate-anticoagulated samples after centrifugation at 2000 × g for 10 min at room temperature using a viscosity-based detection system (Diagnostica Stago; reference range 2.0–4.0 g/l), D-dimer levels were measured by Tina-quant D-Dimer immunoturbidimetric assay, Roche Diagnostics, Mannheim, Germany, reference ≤ 0.5 mg/ml, and prothrombin fragments by F1+2, by a monoclonal enzyme immunoassay (Enzygnost F1 + 2, Siemens Healthcare Diagnostics, reference values 69-229 pmol/l).

4.2.5 Urinary analyses

4.2.5.1 Urinary dipstick tests

In Studies I-III and V, urinary dipstick analyses were carried out by using automated tests based on refractometry; from year 1994 using Miditron M equipment (Roche), from 2004, Urisys 2400 or 1900 (Roche), and from 2009 until 2014, Siemens Clinitec Atlas or Advantus equipment. The sensitivity of dipstick tests (U-Alb-stick) for urinary albumin (1+) ranges from 0.15-0.3 g/l. A dipstick result 2+ represents >1 g/l albumin and 3+, >3 g albumin/l. The test detects urinary albumin but it does not react with immunoglobulins or α_1-microglobulin.

In Studies II and III urinary dipstick haematuria (U-Eryt-stick) was determined by using the aforementioned automated analysers. The dipstick assay for haematuria detects haeme pseudoperoxidase activity and therefore it also detects red cell casts

and dysmorphic red cells. The presence of myoglobin in the urine can give false-positive results. The sensitivity of the assay is approximately 10 x 10^6 cells/l (approximately 3–5 cells per high-power field) and results indicating > 2 erythrocytes /high-power field were considered elevated. Urine sediment erythrocytes were assessed in 189 (92 %) patients either by microscopy or by flow cytometry. An estimate of one erythrocyte/high-power field = 5.8 × 10^6/l was used to standardize the results of these two methods used to assess the sediment.

In Study III, dipstick leukocyturia and glucosuria were also evaluated by using the aforementioned automated analysers on admission. The dipstick test for leukocytes (U-Leuk-stick) detects granulocyte and macrophage esterase activity. The sensitivity is approximately 30 × 10^6 cells/l (1–2 cells per high-power field). The dipstick test for glucose (U-Gluc-stick) detects glucosuria at glucose levels of 3 to 5 mmol/l upward. A U-Gluc-stick reading of 3+ corresponds to a glucose level exceeding 30 mmol/l.

4.2.5.2 Timed urinary excretion of albumin and other proteins

In Studies I and II, timed urinary albumin (cU-Alb), IgG (cU-IgG) and α_1-microglobulin (cU-$\alpha_1$miglo) excretion levels were analysed in 70 patients using urine collected overnight. Urine collection started on the first evening of hospital care and was continued for the next three nights. A 24-hour collection was carried out during the same three days. The volume of urine and the timeframe of the collection were recorded.

In Study IV, overnight cU-Alb was measured once during hospital stay. The measurement was repeated once or twice in the convalescent phase, 10-28 days after hospital discharge (within three months after fever onset), and at six-month control visits. The number of cU-Alb measurements per patient varied from one to three (median two). The time delay between sampling and first symptoms, i.e. fever onset, was recorded.

In Studies I and II, assay of urinary albumin, IgG and α_1-microglobulin was carried out by nephelometry (Behring Nephelometer II Analyzer, Behringwerke AG, Marburg, Germany). To determine 24-hour urinary protein excretion, samples were analysed using the pyrogallol red molybdate method (Olli C.; Kone Instruments, Helsinki, Finland) until 1998 and thereafter by using Cobas Integra (Roche diagnostics) equipment. In Study IV, assay of urinary albumin was carried out by using an immunoturbidometric method on a Cobas C 702 –clinical chemistry analyzer (F. Hoffman – La Roche Ltd., Basel, Switzerland). The detection limit of

the assay for albumin was 3 mg/L. The excretion rate in the urine was calculated by using the volume of urine collected in a recorded timeframe (minutes). cU-Alb, cU-IgG and cU-$\alpha_1$microglobulin were expressed as μg/min and 24-h urinary protein excretion expressed as g/24 h. Excretion of α_1-microglobulin of over 7 μg/min was considered abnormal.

4.2.6 Plasma adipokine and IL-6 analyses

In Study V, plasma samples for assays of the adipokines resistin, leptin and adiponectin, as well as for CRP and IL-6 were stored frozen at -70 °C. Analyses were carried out by using ELISAs with reagents from R&D Systems Europe Ltd., Abingdon, UK (resistin, leptin, adiponectin and CRP) and from eBioscience Inc., San Diego, CA, USA (IL-6). Detection limits and interassay coefficients of variation were 15.6 ng/ml and 8.5 % for resistin, 15.6 ng/ml and 5.3 % for leptin, 15.6 μg/ml and 6.0 % for adiponectin, 3.9 μg/ml and 5.7 % for CRP, and 0.39 μg/ml and 4.8 % for IL-6. Regarding adiponectin, the test detects total adiponectin.

4.2.7 Statistical analysis

For the descriptive analyses, medians and ranges were given for skewed continuous variables and numbers and percentages for categorical variables. The Spearman rank correlations were used to study the relationship between variables. Comparative analysis was performed by using Mann-Whitney U test or Kruskal-Wallis test as appropriate. Related samples were compared by Wilcoxon signed rank test or the Friedman test as appropriate. A logistic regression analysis was performed to assess the associations of age, gender, BMI, U-Alb-stick (0/1+, 2+ and 3+) and maximum plasma resistin level as independent risk factors for severe AKI. Adjusted odds ratio (OR) and their 95 % confidence intervals (95 % CI) are given. All tests were two-sided and p-values < 0.05 were considered statistically significant. The receiver operating characteristic (ROC) curves were generated to evaluate the performance of inflammatory markers to indicate severe AKI. (Figure 5.) The SPSS statistical software package (IBM SPSS Statistics version 23.0 Armonk, NY, USA) was used for all analyses.

5 RESULTS

5.1 Characteristics of study populations (I-V)

The clinical and common laboratory findings of the patients in Studies I-V are shown in Table 6. Patients were admitted to hospital at a median of four (range 1-15) days after the onset of fever. A maximum plasma creatinine level of over 100 μmol/l was detected in 70-75 % of the patients in these Studies. In all five Studies, the hospital care took place mostly in a regular hospital ward and the median duration of hospital stay was six (range 2-30) days. Of all patients in the studies, six were in clinical shock on admission. Nine patients needed transient hemodialysis treatment. Three of them had clinical shock on hospital admission. Of the three with clinical shock and need of dialysis, two were treated in the intensive care unit. One patient (included in all studies) had acute Guillan-Barré syndrome, probably triggered by PUUV infection. This patient was treated in the stroke unit with plasma exchanges. All patients recovered.

Urinary dipstick results in connection with all three dipsticks (for albumin, erythrocytes, and glucose), were available in 195 cases. The numbers of patients in each category of U-Alb-stick 0-1+, 2+ or 3+ for albuminuria, U-Eryt-stick 0, 1+ or 2-3+ for haematuria and U-Gluc-stick 0 or 1-3+ for glucosuria are presented in Table 7.

Table 6. Laboratory and clinical findings in patients in Studies I-V.

Variable median (range)	Studies I & II n=205	Study III n=195	Study IV n=141	Study V n=79
Duration of fever, days	6 (2-19)	7 (3-22)	7 (1-22)	8 (4-15)
Length of hospital stay, days	6 (2-25)	6 (2-30)	6 (2-22)	6 (2-14)
Body weight change during hospital stay, kg	2.4 (0-12.0)	2.5 (0-12.0)	2.1 (0-12.0)	2 (0-11)
Blood haematocrit max	0.44 (0.33-0.60)	0.44 (0.33-0.60)	0.44 (0.33-0.60)	0.44 (0.33-0.60)
Blood platelet count min, $\times 10^9$/l	61 (3-249)	60 (3-249)	61 (8-238)	52 (5-150)
Plasma creatinine max, μmol/l	200 (51-1499)	199 (51-1499)	185 (51-1499)	186 (51-1499)
Plasma creatinine max >100 μmol/l, n (%)	153 (75 %)	145 (74 %)	103 (73 %)	56 (70 %)
Patients with severe AKI[a] n (%)	63 (31 %)	61 (31 %)	44 (31 %)	25 (32 %)
Plasma sodium min, mmol/l	132 (109-142)	132 (109-142)	130 (109-141)	130 (109-139)
Plasma potassium max, mmol/l	4.3 (3.3-5.5)	4.3 (3.3-5.5)	4.2 (3.3-5.5)	4.2 (3.3-5.3)
Blood leukocyte count max, x10^9/l	10.4 (4.2-45.0)	10.6 (4.2-45.0)	10.5 (3.9-45.0)	10.8 (4.2-45.0)
P-CRP max, mg/ml	79 (16-269)	79 (16-269)	79 (16-269)	57 (8-199)
Plasma IL-6 max, pg/ml				11.8 (1.7-66.6)
cU-Albumin at the acute phase, μg/min	median of max values[b] 760 (4-7026) n=70		284 (2.2-6460) n=133	

Abbreviations: max = maximum, min = minimum, CRP = C-reactive protein, IL-6 = interleukin-6, cU = overnight urinary excretion

[a] severe acute kidney injury (AKI) was defined as maximum plasma creatinine ≥353.6 μmol/l during hospital stay [196]

[b] maximum value out of three measurements in each patient

Table 7. Numbers of patients in different categories of dipstick albuminuria, haematuria and glucosuria on hospital admission in 195 PUUV-infected patients.

	U-Alb 0-1+	U-Alb 2+	U-Alb 3+	U-Gluc 0	U-Gluc 1-3+
U-Eryt 0	19	9	1	26	3
U-Eryt 1+	117	31	32	79	8
U-Eryt 2-3+	9	32	38	66	13
U-Alb 0-1+				49	3
U-Alb 2+				71	1
U-Alb 3+				51	20

Abbreviations: U-Alb = urinary dipstick test for albumin, U-Alb-stick; U-Eryt = urinary dipstick test for erythrocytes, U-Eryt-stick; U-Gluc = urinary dipstick test for glucose, U-Gluc-stick

5.2 Associations of urinary findings with disease severity

5.2.1 Proteinuria (I)

The degree of albuminuria was evaluated in 205 patients from the first urine sample by using urinary dipstick tests. Clearly elevated albuminuria (U-Alb-stick ≥2+) was detected in 151 (74 %) of the patients. The patients were divided into three categories according to baseline dipstick albuminuria: U-Alb-stick 0-1+, 2+ and 3+ to discover differences in the severity of the upcoming disease. The results are presented in Table 8. The more the amount of dipstick albuminuria on hospital admission the higher the maximum of plasma creatinine measured during hospitalization. Other markers that reflect overall disease severity, including longer duration of hospital stay, greater weight change, higher haematocrit, lower plasma sodium, higher potassium and higher blood leukocyte counts were also associated with a higher degree of dipstick albuminuria on admission. Minimum plasma albumin values were not associated with the amount of dipstick albuminuria. Maximum CRP values differed between albuminuria groups but showed no association with an increasing amount of albuminuria. Minimum platelet counts did not differ between dipstick albuminuria groups.

Table 8. Clinical and laboratory data of 205 patients divided into three groups according to the amount of urinary dipstick albumin on hospital admission.

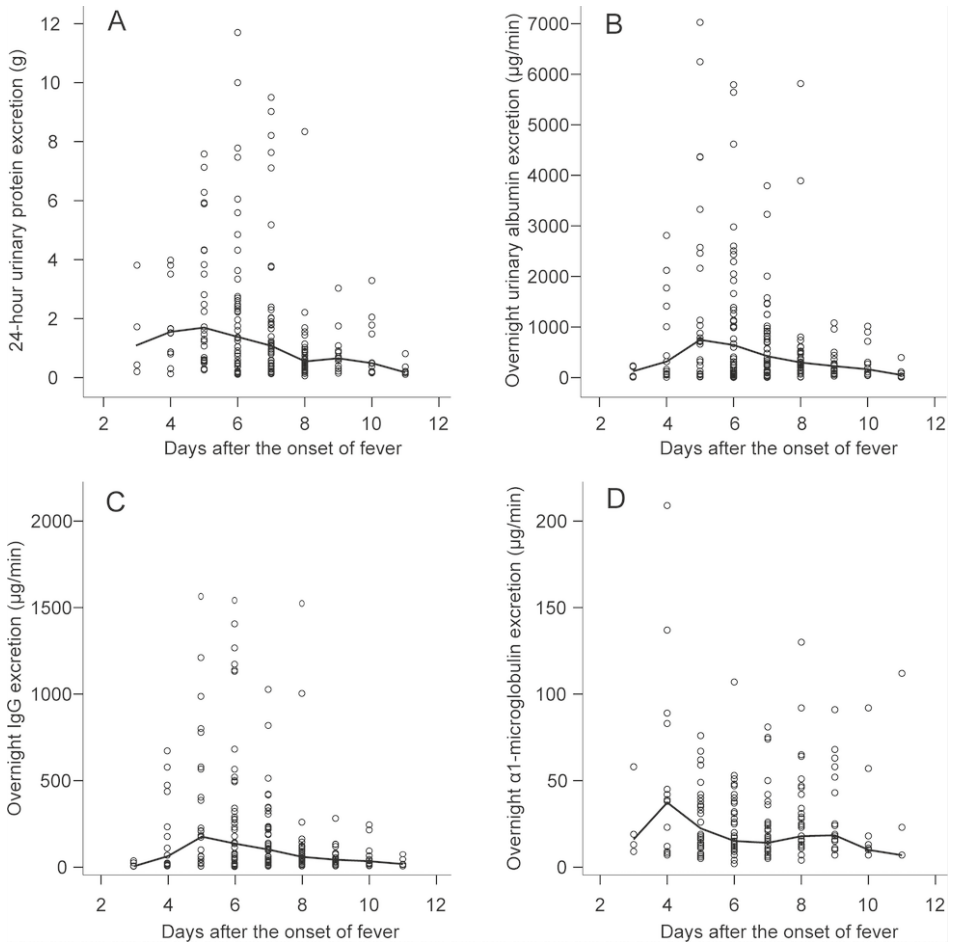
Variable, median (range)	U-Alb 0/1+ n=54	U-Alb 2+ n=73	U-Alb 3+ n=78	p-value
Days from the fever onset to hospital admission	4 (1-8)	4 (1-7)	4 (1-15)	0.465
Length of hospital stay, days	5 (3-22)	6 (3-15)	7 (2-14)	0.026
Body weight change during hospital stay, kg	1.6 (0-10.8)	2.2 (0-10.0)	3.7 (0-12.0)	<0.001
Blood haematocrit max	0.42 (0.33-0.59)	0.44 (0.34-0.59)	0.46 (0.34-0.60)	0.001
Blood platelets min, $\times 10^9$/l	68 (3-249)	61 (15-198)	56 (5-187)	0.232
Plasma creatinine max, μmol/l	98 (52-1447)	139 (71-829)	363 (51-1285)	<0.001
Plasma sodium min, mmol/l	135 (109-141)	133 (120-141)	130 (113-142)	0.001
Blood leukocytes max, $\times 10^9$/l	9.2 (5.1-38.6)	9.0 (4.2-31.2)	13.0 (5.7-45.0)	<0.001
Plasma C-reactive protein max, mg/l	74 (16-236)	92 (20-269)	68 (21-214)	0.012
Plasma albumin min, g/l (n=113)	30 (11-39)	28 (21-37)	27 (18-37)	0.161
	n=12	n=30	n=29	
Urinary protein excretion max, g/24h (n=70)	0.57 (0.14-9.50)	1.74 (0.18-17.78)	2.22 (0.30-10.00)	0.076
Overnight urinary albumin excretion max, μg/min (n=70)	104 (4-4617)	756 (12-6246)	1016 (136-7026)	0.007
Overnight urinary IgG excretion max, μg/min (n=70)	16 (7-1267)	211 (3-1565)	226 (25-1542)	0.005
Overnight urinary α_1-microglobulin excretion max, μg/min (n=70)	20 (10-89)	38 (9-209)	30 (2-130)	0.693

Abbreviations: U-Alb = urinary dipstick test for albumin, U-Alb-stick; max = maximum, min = minimum, IgG = immunoglobulin G

In the subgroup of 70 consecutive patients whose urine was collected during the first three days after admission, the median of maximum 24-h urinary protein excretion was 1.8 g, with wide variation from 0.14 to 17.78 g/24 h between the patients. Nephrotic-range proteinuria (>3.5 g/24 h) was detected in 24 (34 %) of the patients. Tubular proteinuria, elevated urinary excretion of α_1-microglobulin, was present in 90 % of the patients.

The kinetics of urinary excretion of different proteins in relation to the onset of fever in 70 patients with three consecutive days of urine collection is presented in the Figure 3. The greatest degree of 24-h proteinuria was detected on the fifth day after the onset of fever (peak median 1.67 g/24 h, range 0.26-17.78) as shown in the Figure 3a. Urinary excretion of glomerular proteins, i.e. albumin (peak median 734 μg/min, range 12-7026) and IgG (peak median 183 μg/min, range 6-1565) also peaked at the fifth day after fever onset, as shown in the Figures 3b and 3c. Urinary excretion of α_1-microglobulin, considered to reflect tubular proteinuria, peaked at the fourth day (peak median 38 μg/min, range 7-209, Figure 3d). During the 6th to the 9th day the excretion of α_1-microglobulin was more stable compared with the decreasing amounts of albumin and IgG.

Figure 3. Amounts of urinary excretion of proteins over three hospital days in 70 patients according to the time since fever onset.



Every circle represents one measurement, the line depicts median values.

The timing of the plasma creatinine peak from the beginning of fever was determined by the daily creatinine measurements done during hospitalization (median five measurements in each patient, range 2-15). Plasma creatinine peaked at nine days after the onset of fever, four to five days later than the peak excretion of urinary proteins. In 70 patients with analysis of timed urinary excretion analysis, the correlations between maximum creatinine levels vs. maximum overnight excretion of the glomerular proteins IgG ($r=0.44$; $p<0.001$) and albumin ($r=0.41$; $p<0.001$) were statistically significant, while maximum 24-h total urinary protein levels

correlated to a lesser degree with maximum creatinine levels (r=0.30; p=0.012). The maximum amounts of urinary excretion of α_1-microglobulin did not correlate with maximum plasma creatinine values (r=0.12; p=0.291) nor other disease-severity markers except for maximum CRP levels (r=0.39; p=0.001).

Severe AKI, defined by maximum plasma creatinine level of ≥ 353.6 μmol/l was detected in 63 (31 %) of all 205 patients. The sensitivity of a dipstick albuminuria result of ≥2+ to find patients with severe AKI was 89 %, with a specificity of 67 %. An albumin dipstick test result of 3+ had a sensitivity of 62 % in detecting a plasma creatinine level of >200 μmol/l (the median value in this study population) with a specificity of 86 %.

Six patients out of 205 had clinical shock; four of them had a U-Alb-stick test result of 3+, the other two had results of 1+ and 0+. Of nine patients who received dialysis treatment during their hospital care, five had U-Alb-stick test result of 3+, one had a result of 2+ and the others, result of 0 or 1+.

5.2.2 Haematuria (II)

Haematuria, defined as a U-Eryt-stick result of ≥ 1+ was present in 177 (86 %) of the patients, including 83 (40 %) patients with U-Eryt-stick result of 2-3+. Urine sediment, examined in 189 (92 %) patients, showed elevated erythrocyte levels in 122 (65 %) patients.

Dipstick-verified haematuria associated with the severity of AKI. The maximum values of plasma creatinine were significantly greater in parallel with a rising amount of dipstick-verified haematuria categorized as 0, 1+ and 2+/3+ (p=0.001), as seen in Table 9. The amounts of erythrocytes in the sediment showed a slight correlation with maximum creatinine values (r=0.168; p=0.021). A greater degree of dipstick haematuria on admission predicted significantly a greater weight change during hospital stay, lower minimum haematocrit values, and lower minimum plasma albumin and sodium levels during hospital stay. A greater degree of dipstick haematuria was also associated with higher levels of overnight urinary excretion of albumin and total protein during the first days of hospital stay in 70 patients with the aforementioned urine collections, while there was no association between dipstick haematuria and urinary excretion of α_1-microglobulin. The inflammatory markers, blood leukocytes and CRP were not associated with dipstick haematuria either.

The level of thrombocytopenia did not show an association with dipstick haematuria (Table 9) or the number of erythrocytes in the urine sediment (r=-0.124,

p=0.088). The coagulation and fibrinolysis markers were elevated but fibrinogen, prothrombin fragments F1+2 and D-dimer showed no association with the amount of dipstick-expressed haematuria either.

Table 9. Clinical and laboratory findings in 205 patients with PUUV infection categorized by the degree of dipstick haematuria.

Variable, median (range)	U-Eryt 0 n=28	U-Eryt 1+ n=94	U-Eryt 2-3+ n=83	p-value
Hospital stay, days	5.5 (3-22)	6.0 (3-25)	6.0 (2-16)	0.181
Body weight change during hospital stay, kg	1.5 (0-9.4)	2.1 (0-10.7)	2.8 (0-12.0)	0.015
Blood haematocrit min	0.38 (0.25-0.43)	0.36 (0.25-0.43)	0.35 (0.22-0.46)	0.022
Plasma creatinine max, µmol/l	99 (58-874)	175 (52-1499)	265 (51-1285)	0.001
Plasma sodium min, mmol/l	135 (126-139)	132 (114-142)	131 (109-141)	0.005
Blood leukocytes max, $x10^9$/l	9.8 (4.2-38.6)	10.7 (5.4-31.2)	10.4 (4.4-45.0)	0.443
Plasma C-reactive protein max, mg/l	74 (16-236)	75 (20-244)	84 (16-269)	0.436
Plasma albumin min, g/l (n=113)	32 (11-37)	29 (20-39)	26 (18-39)	0.002
dU-Protein max, g/day (n=70)	0.7 (0.3-3.7)	2.6 (0.3-10.0)	1.8 (0.1-17.8)	0.019
cU-Albumin max, µg/min (n=70)	97 (14-738)	987 (14-7026)	738 (4-6246)	0.005
cU-α_1-microglobulin max,µg/min (n=70)	42 (20-89)	21 (7-130)	27 (7-209)	0.280
Urine sediment erythrocytes, /field of view (n=189)	2 (1-132)	3 (0-41)	8 (0-401)	<0.001
Blood platelets min, $x10^9$/l	63 (19-172)	67 (13-249)	56 (3-198)	0.307
Plasma fibrinogen, g/l (n=43)	4.2 (2.0-9.6)	4.2 (2.2-6.7)	4.5 (1.5-5.7)	0.863
Plasma D-dimer, mg/l (n=43)	1.4 (0.6-29.6)	3.4 (0.3-13.5)	3.3 (0.8-34.0)	0.405
Plasma prothrombin fragments F1+2, pmol/l (n=43)	542 (329-1160	583 (149-1487)	711 (284-1875)	0.811

Abbreviations: U-Eryt = urinary dipstick test for erythrocytes, U-Eryt-stick; min = minimum, max = maximum, dU = 24-hour urinary excretion, cU = overnight urinary excretion

5.2.3 Glucosuria (III)

Glucosuria was detected in the dipstick urine sample in 24 of 195 (12.3 %) patients on admission to hospital. The amount was 1+ in 20 patients, 2+ in three patients and 3+ in one patient. Patients with glucosuria on hospital admission were nonglucosuric later according to follow-up samples. The patients arrived at hospital at a median of four days after the onset of fever, with no difference between glucosuric and nonglucosuric patients.

The clinical data of the patients with and without glucosuria are shown in Table 10. Glucosuria was more common in males (15.9 %) than in females (4.8 %). Glucosuric patients had higher BMI, but BMI was not correlated with maximum creatinine values ($r=0.091$; $p=0.231$). Glucosuric patients had significantly higher blood glucose values on admission (available in 39 % of the patients), but none of the glucosuric patients had had a previous diagnosis of diabetes. Patients with glucosuria were in clinical shock on admission more often than nonglucosuric patients. They also had significantly lower first measured systolic blood pressure. Glucosuric patients had longer hospital stays and greater weight change during hospitalization.

Laboratory findings of the patients according to the presence of glucosuria are presented in Table 11. Patients with glucosuria had higher maximum plasma creatinine concentrations during hospitalization as well as higher baseline creatinine levels on admission. When adjusted according to creatinine concentrations on admission, glucosuria remained a significant predictor of severe AKI, defined as a plasma creatinine level of $\geq$ 353.6 μmol/l; (odds ratio 5.9, 95 % CI 1.9-18.0).

The minimum number of platelets was also significantly lower in glucosuric than in nonglucosuric patients (Table 11). Maximum haematocrit values detected were also higher and minimum plasma albumin values lower in glucosuric vs. nonglucosuric patients. Glucosuric patients had significantly higher maximum leukocyte counts during hospitalization, but no difference was found in maximum CRP values compared with nonglucosuric patients.

Table 10. Clinical characteristics of patients with acute Puumala virus infection according to the presence of glucosuria.

	Glucosuria n=24		No Glucosuria n=171		p value
Clinical findings	Median/ number	Range/%	Median/ number	Range/%	
Age, years	40	25-67	41	21-73	0.908
Males, number (%)	21	87.5	111	64.5	0.034
BMI, kg/m^2	28	20-37	26	19-42	0.031
Length of hospital stay, days	7.5	4-22	6	2-30	0.009
Body weight change during hospital stay (kg)	4.0	0.5-11.3	2.5	0.1-12.0	0.025
The first systolic BP[a], mmHg	115	70-167	124	72-210	0.025
Shock on admission, number (%)	5	20.8	2	1.2	<0.001
Need for dialysis, number (%)	3	12.5	7	4.1	0.110

Abbreviations: BMI = body mass index, BP = blood pressure

[a] the first systolic blood pressure measured at emergency room

Table 11. Laboratory variables during hospital stay due to acute Puumala virus infection according to the presence of glucosuria.

	Glucosuria n=24		No Glucosuria n=171		p value
Laboratory findings	Median	Range	Median	Range	
Blood haematocrit max	0.51	0.34-0.60	0.43	0.33-0.59	<0.001
Blood platelets min, x10^9/l	41	5-102	62	3-249	0.006
Plasma creatinine first[a], µmol/l	184	72-617	96	43-1113	0.002
Plasma creatinine max, µmol/l	459	78-1041	166	51-1499	<0.001
Plasma sodium min, mmol/l	128	122-140	132	109-142	0.001
Plasma albumin min, g/l (n=105)	24	11-33	28	20-39	<0.001
Blood leukocytes max, x10^9/l	16.0	5.7-45	10.2	4.2-35.5	<0.001
Plasma CRP max, mg/l	81	21-213	77	16-269	0.446
Blood glucose on hospital admission, mmol/L (n=76)	8.3	4.9-17.6	5.9	3.6-10.9	0.001

Abbreviations: max = maximum, min = minimum, CRP = C-reactive protein

[a] the first plasma creatinine measured at emergency room

5.2.4 Associations of combined urine dipstick results with disease severity (III)

The amount of dipstick-verified haematuria (graded 0-3+) had an association with the amount of dipstick-verified albuminuria (graded 0-3+) ($p<0.001$). The majority of glucosuric patients, 20 out of 24 (83 %) had dipstick albuminuria values of 3+. In non-glucosuric patients, proportion of patients with 3+ albuminuria was 30 % (51 out of 171). The distributions of each category of dipstick haematuria in glucosuric and non-glucosuric patients were 0/1+ 13 % and 15%, 2+ 33 % and 46%, 3+ 54% and 39%, respectively. (Table 7 in page 59).

Those 71 patients with the highest degree of dipstick albuminuria (3+) are presented in Table 12, according to the presence of glucosuria. In these patients, the presence of glucosuria was associated significantly with clinical shock, higher maximum creatinine values, higher haematocrit values, lower minimum blood-platelet counts as well as lower plasma albumin and sodium levels.

Table 12. Clinical variables according to the presence of glucosuria in 71 patients with severe albuminuria (U-Alb-stick 3+) on hospital admission.

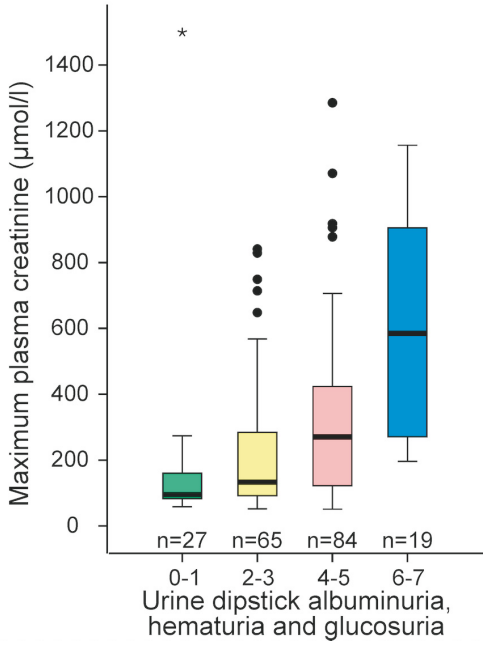
Variable, median (range)	U-Alb 3+/U-Gluc 0	U-Alb 3+/U-Gluc 1-3+	p value
Number of patients	51	20	
BMI, kg/m^2	25 (20-42)	28 (20-34)	0.038
Length of hospital stay, days	7 (2-13)	8 (4-14)	0.123
Body weight change during hospital stay, kg	3.6 (0-12.0)	4.7 (0.9-11.3)	0.261
First systolic blood pressure[a], mmHg	129 (100-210)	115 (88-167)	0.012
Clinical shock, number of patients	0	4	0.001
Need for dialysis, number of patients	4	2	0.770
Haematocrit max	0.44 (0.34-0.59)	0.52 (0.34-0.60)	<0.001
Blood platelets min, ×10^9/L	59 (9-187)	40 (5-102)	0.034
Plasma creatinine max, μmol/L	350 (51-1285)	484 (185-1041)	0.031
Sodium min, mmol/L	130 (113-142)	128 (122-135)	0.050
Blood leukocytes max, ×10^9/L	12.6 (6.1-26.8)	16.5 (8.0-45.0)	0.045
Plasma CRP max, mg/L	64 (21-214)	84 (21-213)	0.085
Plasma albumin min, g/L (n=47)	28 (22-37)	24 (18-33)	0.001

Abbreviations: U-Alb = urinary dipstick test for albumin,U-Alb-stick; U-Gluc = urinary dipstick test for glucose, U-Gluc-stick; BMI = body mass index, max = maximum, min = minimum, CRP = C-reactive protein

[a] the first systolic blood pressure measured at emergency room

When combining the dipstick-test impact, summing the number of positive results (plus-count) of dipstick albuminuria + haematuria + glucosuria on admission, the higher the result, the higher was the maximum plasma creatinine level during the hospital-stay: the median maximum plasma creatinine level was 95 μmol/l (range 58-1499) for plus-count 0 to 1+, 133 μmol/l (range 52-841) for 2-3+, 271 μmol/l (range 51-1285) for 4-5+, and 585 μmol/l (range 196-1156) for plus-count 6-7+. There were no patients with a sum of positive results exceeding 7+. The association between combined dipstick plus-count result and maximum plasma creatinine level during hospitalization is expressed in the Figure 4.

Figure 4. Maximum plasma creatinine values during hospitalization in relation to combined positive results (plus-count) in the urinary dipstick tests on hospital admission.



The numbers of patients in each dipstick test plus-count groups (0-1, 2-3, 4-5, 6-7) are indicated in the figure. Boxplots show the median (thick line), interquartile range (box) and minimum and maximum values within a 1.5 interquartile range (whiskers). Outliers are shown as circles and one extreme value as an asterisk.

5.3 The disappearance of albuminuria after acute Puumala virus infection (IV)

In Study IV, the degree of albuminuria was assessed measurement of overnight excretion of urinary albumin at the acute phase during hospitalization, once or twice during the convalescent phase and at six months in 141 patients. Acute-phase urine sample were obtained in 133 cases. Missing urine samples in cases were a result of either due to anuria or technical issues. In these cases, samples were analysed later at control visits. Higher than normal physiological degrees of albuminuria (cU-Alb >20 µg/min) were found in 116/133 (87 %) of the patients during hospitalization. At the acute phase, the degree of albuminuria was severely increased (cU-Alb 200-1200 µg/min) in 50 (38 %) patients and in the nephrotic range (cU-Alb >1200 µg/min) in 26 (20 %) patients. In the acute phase, median amount of urinary excretion of

albumin measured within seven days after the onset of fever in 77 cases was 311.4 μg/min (range 2.2-6460.7) and measured between 8-13 days in 56 cases, it was 234.9 μg/min (range 6.8-5479.2) (no statistically significant difference; p= 0.695).

Table 13 shows sequential cU-Alb measurements at different time points after the onset of fever, categorized according to the degree of albuminuria in 133 patients during their hospital stay. Control samples from eight patients with missing samples during hospital stay are also shown. All but one of the measurements after 14 days from the beginning of fever showed no significant albuminuria anymore. The one patient with slightly elevated cU-Alb (35.4 μg/min) at day 44 after the onset of fever had a maximum plasma creatinine level 138 μmol/l and a missing urine sample at the acute phase. This patient did not take part in the other follow-up visit either. The other seven patients with a missing acute-phase sample, did not show albuminuria at the control visits; all had cU-Alb < 20 μg/min. There were patients with severe AKI in each category of the amount of albuminuria in the acute phase, as presented in Table 13. Among 44 patients with severe AKI, cU-Alb was controlled within 28 days from fever onset in 43 cases and none of them showed significant albuminuria subsequently.

Table 13. Albuminuria in control measurements at different time points categorized according to the degree of albuminuria during hospital stay in 141 Puumala virus-infected patients.

			Days after the beginning of fever				
cU-Alb categories at the acute phase	Number of patients with severe AKI[a]	cU-Alb (µg/min) during hospital stay median (range)	14-20 median 19 days	21-30 median 24 days	31-40 median 38 days	41-60 median 46 days	6 months
	n=42 (% of the patients on the row)	n=133	cU-Alb (µg/min) at control visits, median (range) n=41	n=31	n=24	n=44	n=34
<20 µg/min	n=1 (5 %)	n=17 11.4 (2.2-19.0) at median 6 days (4-13)	n=7 3.5 (2.4-9.0)	n=4 2.1 (1.9-2.5)	n=4 2.0 (1.0-4.6)	n=3 1.5 (0.8-1.8)	n=6 1.8 (1.4-6.9)
>20-200 µg/min	n=12 (30 %)	n=40 86.4 (21.4-198.4) at median 8 days (3-12)	n=19 2.2 (0.5-14.6)	n=5 1.8 (0.4-8.0)	n=10 2.4 (0.7-6.3)	n=15 3.2 (0.2-5.4)	n=9 1.5 (1.1-5.4)
>200-1200 µg/min	n=19 (38 %)	n=50 410.1 (203.1-974.8) at median 7 days (3-13)	n=8 4.6 (0.8-14.5)	n=14 2.4 (0.5-18.4)	n=7 1.8 (0.5-7.8)	n=19 2.6 (1.0-7.8)	n=11 2.6 (0.6-14.5)
>1200 µg/min	n=10 (38 %)	n=26 1981.1 (1202.5-6460.7) at median 6.5 days (4-10)	n=7 3.8 (1.3-18.2)	n=8 4.9 (1.0-8.0)	n=3 2.5 (2.1-10.1)	n=7 3.3 (1.7-14.3)	n=8 2.0 (1.3-3.1)
missing urine sample	n=2 (25 %)	n=8 -	n=0 -	n=1 3.1	n=3 1.9 (0.3-2.2)	n=3 2.7 (1.1-35.4)	n=2 3.3 (2.3-4.3)

Abbreviations: cU-Alb = overnight urinary excretion of albumin

[a] severe AKI defined as maximum plasma creatinine ≥ 353.6 µmol/l during hospital stay

5.4 Plasma adipokines and their associations with disease severity and other inflammation markers (V)

Plasma adipokine measurements during hospitalization were carried out in 79 cases median 2 times (range 1-5) and median 7 days (range 3-14) after the onset of fever. Table 14 shows plasma levels of three different adipokines in the acute phase, at the first control visit at a median of 15 days (range 7-21) after discharge from hospital, and at one year. When comparing acute-phase plasma levels with the later measurements, the changes already showed a return near to the one-year plasma levels in the recovery phase. Plasma resistin values were significantly higher and leptin and adiponectin values lower in the acute phase of the infection compared with those at the control visits.

Table 15 shows the correlations between resistin, adiponectin and leptin levels with disease-severity markers. Maximum plasma resistin levels correlated with maximum creatinine values and other disease-severity markers, while the levels of the other adipokines did not.

Table 14. Plasma levels of adipokines resistin, leptin and adiponectin in the acute phase of the infection, in the recovery phase and after one year in 79 patients.

	Acute phase* (n=79)		Recovery phase (n=74)		After 1 year (n=67)		p value
	median	range	median	range	median	range	
Plasma resistin (ng/ml)	28	11-107	17	7-36	14	7-31	<0.001
Plasma leptin (ng/ml)	5.3	1.2-48.4	12.2	1.6-68.7	12.1	1.8-84.0	<0.001
Plasma adiponectin (μg/ml)	3.76	0.23-10.66	4.07	0.62-10.25	4.36	0.80-13.49	<0.001

*Acute phase values are maximum (resistin) or minimum (leptin, adiponectin)

Table 15. Correlations between plasma adipokine levels and disease-severity markers.

	Plasma resistin max		Plasma adiponectin min		Plasma leptin min	
	r	p value	r	p value	r	p value
Length of hospital stay	0.507	<0.001	0.004	0.970	0.142	0.213
Body weight change during hospital stay	0.433	<0.001	-0.017	0.888	0.002	0.988
Systolic blood pressure on admission	-0.257	0.022	-0.240	0.033	-0.103	0.368
Blood platelets min	-0.254	0.024	-0.085	0.456	0.106	0.352
Plasma creatinine max	0.633	<0.001	0.029	0.802	-0.044	0.700
Plasma sodium min	-0.368	0.001	-0.015	0.895	0.241	0.032
Plasma potassium max	0.369	0.001	-0.004	0.971	0.038	0.742

Abbreviations: min = minimum, max = maximum

Correlations between plasma adipokine levels and other inflammatory markers, (maximum blood leukocytes, plasma CRP and IL-6 values and maximum creatinine values) during hospitalization are shown in Table 16. Plasma maximum resistin levels correlated with maximum leukocyte counts and maximum plasma IL-6 values, while plasma minimum adiponectin had a reverse statistically significant correlation with CRP and IL-6 levels. As seen in Table 14, changes in the adiponectin levels at the acute phase were modest and they did not show significant correlations with the markers of clinical severity (Table 15). Plasma leptin concentrations did not correlate with values of other inflammatory markers (Table 16).

Table 16. Correlations between plasma adipokine concentrations and values of other inflammatory markers during the acute phase of PUUV infection in 79 patients

	Plasma resistin max		Plasma adiponectin min		Plasma leptin min	
	r	p value	r	p value	r	p value
Blood leukocytes max	0.520	<0.001	0.060	0.600	-0.078	0.495
Plasma IL-6 max	0.329	0.003	-0.270	0.016	-0.160	0.160
Plasma CRP max	-0.071	0.536	-0.457	<0.001	-0.034	0.763
Plasma adiponectin min	0.081	0.476			0.122	0.284
Plasma leptin min	0.009	0.938	0.122	0.284		

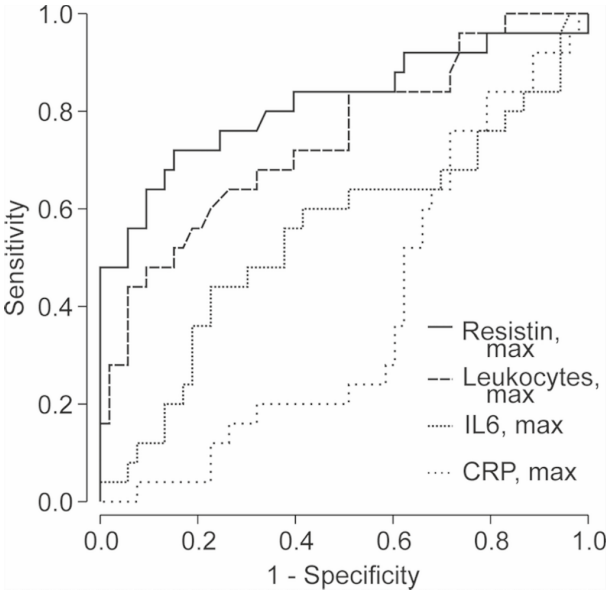
Abbreviations: max = maximum, min= minimum, IL-6 = Interleukin-6, CRP = C-reactive protein

On hospital admission, U-Alb-stick 3+ albuminuria was found in 30 (38 %) and U-Eryt-stick ≥ 2+ haematuria in 34 (43 %) of the patients. Plasma maximum resistin levels were significantly higher in patients with U-Alb-stick 3+ compared with the patients with less or no albuminuria in the dipstick test (resistin 39.6 ng/ml, range 11.0-107.3 vs. 25.0 ng/ml, range 11.6-90.7, p=0.001). The combined plus-count of urinary dipstick albuminuria and heamaturia on admission was elevated (5+ or 6+) in 18 patients and those patients had significantly higher maximum resistin values during hospitalization than the other patients with lower simultaneous albuminuria and haematuria (resistin maximum median 42.7 ng/ml, range 16.8-107.3 vs. 25.1 ng/ml, range 11.1-90.7, p<0.001).

To test the ability of maximum resistin to identify patients with severe AKI (i.e. maximum plasma creatinine ≥ 353.6 μmol/l during hospital stay) compared with other inflammation markers studied, receiver operating characteristic (ROC) curves were generated for the maximum plasma resistin, blood leukocyte counts, and plasma IL-6 and CRP levels. Resistin was a stronger discriminator, with an area under the ROC curve (AUROC) of 0.82 (95 %CI 0.70-0.91, p<0.001) when compared with leukocyte count (AUROC 0.74, 95 % CI 0.62-0.87; p=0.001) as seen in the Figure 5. Neither IL-6 nor CRP discriminated severe AKI from non-severe AKI (AUROC 0.55, 95 % CI 0.40-0.70; p=0.493 and AUROC 0.39, 95 % CI 0.26-0.51; p=0.105, respectively).

In the univariate model analysis, male gender, 3+ dipstick albuminuria and maximum plasma resistin were associated with severe AKI. In the multivariable model, only male gender and maximum plasma resistin were found to be independent risk factors of severe AKI.

Figure 5. Receiver operating characteristic (ROC) curves for plasma inflammatory markers maximum resistin, maximum C-reactive protein, maximum interleukin-6 and maximum blood leukocyte count, in predicting severe AKI (plasma creatinine maximum ≥ 353.6 μmol/l) during hospitalization.



Abbreviations: max = maximum during hospital stay, IL6 = Interleukin-6, CRP = C-reactive protein

6 DISCUSSION

6.1 Associations of urinary findings with disease severity in acute Puumala virus infection

The Studies I, II, and III showed that the amounts of dipstick albuminuria and haematuria and the presence of glucosuria on admission predict the severity of upcoming AKI during hospitalization in patients with acute PUUV infection. The higher the total plus-count of these dipstick urinary findings together were, the more severe was subsequent AKI. The presence of glucosuria also associated with clinical shock and the minimum platelet count. Study IV further showed that no significant albuminuria remained at 2-3 weeks after fever onset. The disappearance rate was not affected by AKI severity or the amount of albuminuria in the acute phase of the disease.

6.1.1 Dipstick proteinuria and haematuria in Puumala virus infection

Study I showed that a higher amount of dipstick albuminuria on hospital admission was associated with the severity of AKI during hospitalization. A retrospective German study has previously shown that proteinuria on admission could predict the severity of upcoming AKI in PUUV-infected patients [150]. In that study, the urinary protein/creatinine-ratio, the urinary albumin/creatinine ratio and/or dipstick proteinuria were measured in 61 PUUV-infected patients. All the markers of proteinuria correlated with peak creatinine values. Dipstick protein ≥2+, corresponding 1-3 g/l, was found in 58 % of patients. The delay from the onset of symptoms to hospital admission was median six days, which is two days more than in Study I, pointing out that the presence of albuminuria on admission had a predictive value although the test was obtained almost a week after the onset of symptoms. To conclude, Study I confirmed the finding that dipstick albuminuria test on admission is useful in predicting upcoming AKI in patients with acute PUUV infection.

Factors related to severe disease has previously been investigated in retrospective analysis of 205 French patients with acute PUUV infection [60]. Severe disease was defined by one of the following: shock symptoms, urine output <300 ml/d, plasma creatinine level >353.6 μmol/l, need of dialysis, ICU treatment or blood transfusion. Severe disease was found in 22 % of the patients. Haematuria on admission was present in 47 % of 199 patients, mean 5.3 days after fever onset and the presence associated with severe disease in univariate model. Urine dipstick proteinuria on admission was examined in 37 % of all patients and no association of dipstick proteinuria with disease severity was found. The presence of glucosuria was not addressed. [60] The median level of maximum creatinine was 198 μmol/l, peaking on average at 8th day after fever onset, as also observed in patients in Study II. Only the categorical presence of dipstick haematuria, not the amount of dipstick haematuria, was included in the analysis. Thus, the results of Study II confirm this finding that the presence of haematuria on admission is associated with severe PUUV-induced AKI. Study II also showed that the association of dipstick haematuria with AKI severity is dependent on the amount of haematuria.

6.1.2 Possible mechanisms of proteinuria and haematuria

The incidence of proteinuria up to nephrotic range (>3,5 g/d) in 30 % of PUUV-infected patients is exceptional when compared with other forms of ATINs, usually characterized by mild proteinuria, under 1 g/day [197]. In the literature, case reports about AKI with sudden nephrotic-range proteinuria are associated with the use of non-steroidal anti-inflammatory drugs. Kidney insufficiency in these cases is usually transient and clinically the condition resembles minimal change nephropathy, although the kidney-biopsy findings can indicate ATIN [198]. In Study I, the peak of glomerular proteinuria occurred early in the course of the disease, clearly preceding the peak values of plasma creatinine. What explains the association of the amount of glomerular proteinuria with decrease in GFR in PUUV infection is unresolved. This kind of association has not been documented in any other acute kidney disease. Decrease in GFR seems to be caused by mechanisms other than sole glomerular endothelial cell damage or clear glomerular morphological injury.

In Study IV, the nature of flash-like albuminuria fits with the observations of reversible podocytopathy present in PUUV infection suggested by a few earlier findings in kidney-biopsy samples [17, 106]. The latest German study showed an association between high levels of urinary nephrin as a marker of podocyte injury,

and high amount of glomerular proteinuria in 26 patients [199]. Podocyte foot-process effacement was documented in all three patients with a kidney-biopsy sample. The study further underlines the similarities between PUUV infection and idiopathic nephrotic syndromes (INSs) with similar correlations of urinary nephrin levels and glomerular proteinuria. The association of higher proteinuria with higher serum creatinine in PUUV infection was also confirmed in this German study, not a typical finding in INS which usually manifests without AKI. [199] In some forms of podocythopathies, a yet unknown circulating factor inducing filtration barrier defect is presumed to be present [200]. In podocyte biology, numerous proteins are involved in cytoskeleton formation, cell contacts with basement membrane and between other podocytes, and some of these interactions are mediated by β_1-integrins [201]. Whether or not any of the host immune-response factors or the virus itself could have an effect on transient podocyte structural and functional alterations in PUUV infection remains unknown.

Although the renal histological findings in PUUV infection are mainly described as acute tubulointerstitial nephritis, a commonly used urinary marker of tubular damage, the rise in urinary excretion of α_1-microglobulin, was not associated with higher maximum plasma creatinine values, nor with the amount of albuminuria or haematuria in Studies I and II. Hence, the results of these studies suggest that the degree of kidney injury in PUUV infection is not well characterized by the amount of urinary excretion of α_1-microglobulin. As a conflicting result, a recent Swedish study showed a slight positive correlation of the urinary α_1-microglobulin/creatinine ratio with plasma creatinine concentrations in a cohort of 39 patients [202]. In oliguric patients with AKI there is a possibility, that rapidly altered GFR and tubular functions can affect the amounts of components measured in the urine hourly, possibly explaining the conflicting results between samples analyzed from timely collected urine and spot-urine adjusted with urinary excretion of creatinine. An optimal way to measure in AKI is not known.

The mechanisms of haematuria in PUUV-induced HFRS are mainly unclear. Renal biopsy findings show tubulointerstitial inflammation, interstitial haemorrhages and peritubular capillaritis. Medullary haemorrhages are typical findings seen in 20-60% of acute phase-biopsy samples [16, 17]. The amount of haematuria, however, has not been associated with the extent of medullary haemorrhages [16]. The presence of dysmorphic red-blood cells in the urine suggests a glomerular origin but the presence of these cells in patients with PUUV infection has not been studied. In Study II, the amount of dipstick haematuria did not associate with markers reflecting blood coagulation abnormalities or the degree of thrombocytopenia. In other forms

of ATIN, the significance of haematuria have been analysed in 110 patients with biopsy-proven ATIN without glomerular diseases. Haematuria was a frequent finding, detected in 60 % of patients, and associated with the amount of albuminuria as well as higher serum creatinine level at the end of follow-up [97]. Hence, haematuria seems to be a frequent finding among patients with ATIN, although the underlying mechanisms explaining haematuria remains to be elucidated.

6.1.3 Role of urinary findings in acute kidney injury due to reasons other than Puumala virus infection

In the literature, there are hardly any reports about the role of de novo albuminuria predicting upcoming AKI or its severity in other conditions than acute PUUV infection. Some investigational data concerning the presence of new-onset albuminuria and its predictive role in kidney outcome in the more common forms of AKI exist. In a paediatric population, among patients undergoing cardiac surgery, a minor increase in the postoperative urinary albumin-creatinine ratio, presumably new-onset albuminuria, has been associated with an increased risk of postoperative AKI [203]. In 423 critically ill patients with sepsis, an association between of *de novo* albuminuria and severe AKI was also found, with new-onset dipstick albuminuria detected in 46 % of the patients [204].

Measurement of proteinuria and hematuria can be performed in several ways. Semiquantitative dipstick albuminuria and hematuria are inexpensive and easy methods to assess urinary findings when large populations are studied, although the results can be regarded as suggestive. In a cohort of over 2400 adult patients with severe burn injuries, the amount of dipstick proteinuria at presentation was associated with the risk of AKI, especially in 396 patients with severe burns. Among those with severe burns, 68 % had dipstick proteinuria and 38 % developed AKI. The patients with previously known kidney insufficiency, diabetes or hypertension were excluded from the study but it was not possible to exclude pre-existing kidney diseases with pre-existing proteinuria. [205] The predictive ability of an increase in dipstick proteinuria in an adult population was analyzed in a study including over 1100 patients who underwent abdominal-aorta surgery. In postoperative serial dipstick samples obtained every six hours during first 24h, an early (<6h) higher change in dipstick proteinuria and urine albumin concentration compared with pre-operative values, was predictive of kidney outcome, defined as need of dialysis or doubling of serum creatinine levels from baseline values, seen in 4,7% of the patients.

The discrimination ability of albuminuria was better compared with urine or serum NGAL, and urine IL-18 [206].

In Studies II and III, albuminuria, hematuria and glucosuria together seemed to have additive effect on their associations with higher maximum plasma creatinine values in PUUV infection. Previously, the predictive role of combined urinary dipstick tests for proteinuria and hematuria was evaluated among over 1800 severely ill patients, with various disease etiologies, treated in ICUs in South Korea. [207] A quarter of the patients had ≥ 2+ proteinuria and 52 % had ≥ 2+ hematuria at ICU admission. The risk of AKI increased gradually with the rising amounts of proteinuria and hematuria together. Also, the risk of 3-year-mortality was significantly associated with dipstick proteinuria and hematuria. The association with increased mortality was found even in patients without AKI. Underlying kidney disease in 9 % of the patients and diabetes in 12 % of the patients were reported. The predictive value of dipstick tests also remained significant in multivariate analysis when adjusted with possible confounding factors. [207] Overall, the data suggest that urinary dipstick proteinuria and hematuria could be worthy of further studies including ICU patients with and without earlier kidney diseases.

6.1.4 Glucosuria, capillary leakage and disease severity

Glucosuria is a rare finding in acute PUUV infection, found in 12 % of patients in Study III. The presence of dipstick glucosuria was associated with more severe disease. Several markers of capillary leakage such as high haematocrit, low first blood pressure and the presence of clinical shock were associated with the presence of glucosuria. Glucosuric patients also had a high frequency of 3+ dipstick albuminuria (83 %). It is thefore possible that glucosuria and albuminuria are related to mechanisms underlying capillary leakage in PUUV infection.

The clinical syndrome of capillary leakage covers conditions with loss of protein-rich fluid from the intravascular to the interstitial space. The conditions that can result in capillary leakage syndrome include, for example, sepsis, snake bites, poisonings (risin), ovarian hyperstimulation syndrome, haemophagocytic lymphohistiocytosis (HLH) and conditions after haematopoietic stem-cell transplantation. It is also related to the use of some drugs, including interleukins, monoclonal antibodies and gemsitabine. Accompanying AKI is typically seen, and a few reports concerning urinary findings exist, covering nephrotic-range proteinuria related to HLH, along with haematuria and proteinuria in risin poisoning [208].

However, PUUV patients without any clinical signs of capillary leakage can still have proteinuria of several grams per day.

Rhabdomyolysis is frequently reported to be accompanied by capillary leakage syndromes, but no cases of rhabdomyolysis are reported in patients with HFRS. It is still not quite impossible that myoglobin in the urine might partly explain the dipstick hematuria findings. However, a previous study showed that serum creatine kinase values didn't indicate rhabdomyolysis in 70 patients with acute PUUV infection [209]. Hematuria was assessed also by means of sediment analysis in Study II, which showed hematuria in 65 % of the patients, although haematuria analysed by this method was only slightly associated with maximum plasma creatinine. The mechanisms by which glucosuria is perhaps associated with capillary leakage are subjects of further studies.

In Study III glucosuria was found in 24 patients. Of those 14 patients with also blood glucose measurement available on admission, two had levels over 10 mmol/l, so glucosuria was not normoglycaemic in all 24 patients. The median blood glucose levels of both glucosuric and nonglucosuric patients on admission were under 10-11 mmol/l, which is considered as a normal tubular threshold-level in healthy, non-pregnant persons and the threshold level can be even higher in CKD. Disturbed tubular function related to AKI and ATIN, with alteration of sodium-glucose cotransporter type 2/glucose transporter 2 (SGLT-2/GLUT2) function in proximal tubules may explain glucosuria [210]. In ATIN caused by other reasons, glucosuria is sometimes encountered in drug-induced ATIN [211]. In children, glucosuria is a common finding in TIN and uveitis (TINU) where impaired kidney function is often acute [99]. In adults, TINU is extremely rare.

The incidence of normoglycaemic glucosuria in other acute virus infections is not known. Signs of proximal tubular dysfunction have been reported in HIV positive persons, which might also be related to antiretroviral medication [212]. HIV nephropathy with glomerular lesions typically represent with interstitial inflammation, distinguishing it from idiopathic collapsing type of focal segmental glomerulosclerosis (FSGS). This type of nephropathy is characterized by proteinuria and progressive CKD, not with reversible AKI [213].

Recently, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was detected in tubular cells by the electron microscopy [214]. Signs of proximal tubule injury i.e. Fanconi syndrome, including possible presence of normoglycemic glucosuria, have been detected in 75 % of 40 patients with SARS-CoV-2 infection. Normoglycemic glucosuria was detected in 9 (35 %) of the ICU patients, while it was present in two (15 %) patients treated in the regular ward [215]. The exact

association of normoglycemic glucosuria with AKI severity was not possible to evaluate even though Fanconi syndrome was suspected to be related to more severe disease. Whether glucosuria can be associated with the virus itself or the overall severity of the disease and kidney injury remains unknown. There is no data on glucosuria incidence or significance in the severely ill to compare the virus-infection-induced AKI with other forms of AKI.

Although the presence of glucosuria is documented in some other forms of AKI and some infections, Study III showed an association of glucosuria with the severity of AKI in PUUV infection. As seen with albuminuria, quick onset and rapid decline, there also seem to be quick alterations in the mechanisms underlying the presence of glucosuria, which was absent in all glucosuric patients in the control samples during hospitalization in Study III. To conclude, in the forthcoming studies on the significance of dipstick findings in other types of AKI, serial determinations could better show possible transient findings.

6.1.5 Clinical utility of urine dipstick test

The frequency of urinary findings, i.e. albuminuria, haematuria and glucosuria in the present cohort of patients corresponds with previous reports [16, 58, 60, 133]. Also, median values of plasma creatinine levels (185-200 μmol/l) in these Studies are close to the values in a previously studied large cohort of 556 patients treated at Tampere University Hospital with a median level of maximum creatinine of 219 μmol/l and to values in a cohort of 355 Swedish patients [12, 58]. The timing of the maximum plasma creatinine values, detected around the 9th day after fever onset, was also congruent with previous reports [12, 37]. The present cohort, therefore, was comparable with previous large, hospital-treated cohorts of patients with acute PUUV infection and the conclusions of these Studies can represent cohorts of hospital-treated PUUV-infected patients.

Pre-existing albuminuria is a well-known risk factor for AKI in general population and in patients with CKD [216]. None of the patients in the present Studies had suffered from any known CKD prior to PUUV infection. Overall, they had quite few underlying diseases. Some of them had previous diagnosis of conditions known as risk factors for AKI, like hypertension in 17 subjects, coronary artery disease in seven and diabetes in five patients. In theory, the presence of previous diseases might influence on outcome of PUUV-induced AKI. However, in a large cohort of 556 patients with acute PUUV infection, there was no difference

in presence of severe AKI between patients with or without chronic diseases prior to infection [12].

Definition of the overall severity of the HFRS is not congruent in PUUV infection studies. Many of the variables used can be regarded as being associated with the kidney disease severity, such as markers reflecting disturbances in fluid balance and electrolytes associated with notable weight changes during hospitalization and longer hospital stays required to regain the balance. Treatment actions are likely to affect the outcome. Plasma creatinine as a sole AKI severity marker, has certain widely recognized limitations in the presence of rapidly changing GFR [217, 218].

The lack of markers reflecting emerging kidney injury and acute tubular damage makes it difficult to recognize patients at risk of severe AKI, disabling the decisions concerning whether or not to start more intensive observation or treatment actions. As the pathogenesis of AKI is unraveled, specific treatment options are difficult to find. Patients with acute PUUV infection are admitted to hospital at different phases of the disease, some with already decreasing amounts of proteinuria and/or stable or decreasing plasma creatinine levels. Hence, the predictive capacity of urinary dipstick findings will not necessarily work in clinical practice, at least not with every patient. As there is great variation of disease severity in PUUV infection, urinary dipstick samples taken at the early phase of the disease, showing only a few plusses, could predict a higher probability of milder disease.

6.2 Plasma resistin and other adipokines in acute Puumala virus infection

Obesity is considered to be an independent risk factor of AKI and the correlation appears linear in critically ill patients [219]. However, in ICU-treated patient populations the effect of BMI on mortality forms a J-shaped curve, so that in the obese patients with BMIs of 30-35 kg/m^2 seem to have a survival benefit compared with normal-weight and underweight patients, and this is called obesity paradox [219]. The increased risk of AKI is very likely to be mediated by the obesity-related comorbidities that also affect kidney health. The endocrine effects of adipose tissue or the obesity-related low-grade inflammation as predisposing factors for an increased risk of AKI cannot be excluded [220]. If the endocrine function of adipose tissue in acute inflammation differs between individuals, previously quite healthy

PUUV-infected patients could be the population in which to explore the effects of adipose-tissue functions on disease and AKI severity.

In Study III, higher BMI did not have a correlation with maximum plasma creatinine values of the patients. In Study V, BMI itself was not clearly associated with adipokine plasma-level changes, excluding a slight correlation of lower leptin levels with higher BMI. Leptin levels rise when there is weight gain, but daily variation is also influenced by the lowering effects of nausea and fasting [221]. Symptoms of overall illness in acute PUUV infection are likely to affect appetite. The explanation for the association of lower minimum leptin levels with lower BMI in Study V remains unsolved but can be explained partly by decreased food intake.

In Study V, higher plasma levels of resistin had a clear association with the severity of AKI and also with the higher amounts of dipstick albuminuria in patients with acute PUUV infection. Higher plasma resistin levels correlated with higher plasma IL-6 levels. Plasma IL-6 has previously also shown good correlation with overall disease severity including AKI severity [108]. Urinary IL-6 has also been shown to be associated with the amount of albuminuria. [95] The amount of urinary IL-6 did not correlate with its plasma concentrations which could be explained by its' local production in the kidneys. Therefore, it might be interesting to study the amount of urinary resistin and its correlations with plasma concentrations as well as AKI severity in PUUV infection. There are, however, methodological problems in the laboratory determination of resistin in the urine, associated with simultaneously high amounts of proteins in the urine in PUUV patients.

The rise in plasma resistin is probably affected by a decrease in its filtration along with decreasing GFR in acute PUUV infection. Some of the PUUV-infected patients with the most severe stage of AKI in Study V had plasma resistin concentrations far higher than those reported in CKD. As resistin was also associated with the amount of albuminuria, the kidneys as possible sites of increased production in PUUV infection cannot be excluded. When higher amount of albuminuria correlate with higher plasma creatine values, whether resistin associate with both or just the other one cannot be specified by this Study.

Male gender along with plasma resistin were risk factors of severe AKI in multivariable analysis. Kidney disease severity measured by the level of plasma creatinine is influenced by the muscle mass, probably accounting for higher maximum creatinine values seen in males. There are no validated, clinically appropriate means to standardize the differences in muscle mass or altered muscle metabolism among patients with AKI. When GFR is changing quickly, as it does in

AKI, a surrogate creatinine-based estimated GFR calculation developed to adjust for the effect of age and gender cannot be used. [222]

In recent years, numerous investigators have tried to find better AKI markers than plasma creatinine. Resistin seems to be one of the markers of immunoactivation, related to macrophage activation [223] and could play a role also in PUUV infection related AKI. There are some studies on the association of resistin level with organ failure and severe sepsis in ICU patients, but studies on associations with AKI severity specifically are lacking. [164, 224] The role of resistin in the pathogenesis of PUUV infection needs further investigations.

6.3 Future considerations

Urinary dipstick tests for erythrocytes give positive results also for red cell casts and dysmorphic red cells which could be present in the urine of PUUV patients. The presence of dysmorphic erythrocytes or the composition of possible casts in urine have not been studied in acute PUUV infection. High amounts of different proteins in urine in acute PUUV infection and urine acidity as well as the effect of sample freezing is a challenge in many laboratory determinations concerning urine in study protocols. Maybe in the future, progress in analyses of urine by means of metabolomics could probably help to find new urinary markers and understand pathogenesis by enabling "metabolic profiling" or "fingerprinting" of various diseases. [225]

Glucosuria is associated with clinical shock and signs of capillary leakage in PUUV infection. There are very few reports about urine findings in hantavirus infections causing HPS, where capillary permeability disturbances occur most clearly in the lungs, leading to acute respiratory distress syndrome. The prevalence and significance of early urine findings in HPS is a subject for future studies as well as the prevalence of glucosuria and its possible role as a sign of ongoing pathogenesis in other severely ill patients.

The mechanism behind prominent polyuria and its high prevalence in PUUV infection-related AKI is not fully understood, although the concentration defect indicates distal tubular damage. In transgenic animal models producing an excess of bradykinin in the kidney proximal-tubule cells, there is an increase of urinary volume and inhibition of the effect of vasopressin in distal tubules. [226] Kallikrein-kinin -system activation and endothelial permeability change induced by HTNV and ANDV with concomitant bradykinin release *in vitro* suggest a possible pathogenic

role of bradykinin, as do also a few case reports concerning icatibant use in PUUV infection. This is an interesting subject for further studies.

7 SUMMARY AND CONCLUSIONS

The main findings of the current study can be summarized as follows:

I The degree of albuminuria in the dipstick tests on hospital admission predicted the severity of upcoming AKI in PUUV infection. Overnight urinary excretion of albumin and IgG as well as total daily urinary protein excretion were associated with AKI severity, while tubular proteinuria, i.e. overnight urinary excretion of α1-microglobulin, was not.

II The degree of haematuria in dipstick tests on hospital admission associated with the severity of AKI during hospitalization, while it was not associated with thrombocytopenia or markers of coagulation or fibrinolysis in acute PUUV infection. Higher amount of dipstick-verified hematuria was associated with higher amount of dipstick-verified albuminuria but not with higher tubular proteinuria during hospitalization.

III Glucosuria was detected in 12 % of the patients by dipstick tests on hospital admission. Its presence was associated with AKI severity and lower blood thrombocyte counts, as well as with clinical shock as a sign of more severe capillary leakage. Higher combined plus-counts in urinary dipstick tests for albumin, erythrocytes and glucose on admission, were associated with higher plasma creatinine values during hospitalization.

IV Albuminuria in acute PUUV infection returns to normal within 2-3 weeks after fever onset and the disappearance rate is not affected by AKI severity or the degree of albuminuria during the acute phase.

V Levels of plasma adipokine resistin were elevated during acute PUUV infection and were associated with AKI severity and the degree of dipstick albuminuria. Slight changes in plasma concentrations of the other adipokines investigated, leptin and adiponectin, did not show significant associations with disease severity.

In conclusion, the degree of dipstick-verified albuminuria and hematuria and the presence of glucosuria on hospital admission are associated with AKI severity in acute PUUV infection. In the case of albuminuria and hematuria, the association with AKI severity seems to be amount-dependent. The presence of glucosuria can help to identify patients at a higher risk of life-threatening disease, as it associated with signs of more severe capillary leakage. Albuminuria in acute PUUV infection shows sudden onset followed by rapid disappearance, which was documented in all patients within 2-3 weeks. Its disappearance was not affected by AKI severity or the amount of albuminuria at the acute phase. This flash-like appearance of albuminuria resembles findings seen in podocythopathies rather than in other instances of acute tubulointerstitial nephritis.

Elevation of plasma levels of adipokine resistin is part of the upregulated immune response detected in acute PUUV infection. The higher resistin level was associated with the severity of AKI as well as with a higher degree of dipstick-verified albuminuria on hospital admission.

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PUBLICATIONS

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PUBLICATION I

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Glomerular Proteinuria Predicts the Severity of Acute Kidney Injury in Puumala Hantavirus-Induced Tubulointerstitial Nephritis

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Keywords
Acute kidney injury · Acute tubulointerstitial nephritis · Albuminuria · Hantavirus · Proteinuria · *Puumala virus*

Abstract
Background: *Puumala virus* (PUUV)-induced hemorrhagic fever with renal syndrome is common in many European countries. The typical renal histologic lesion is acute tubulointerstitial nephritis. We examined the type and kinetics of urine protein excretion and prognostic significance of proteinuria for the severity of acute kidney injury (AKI) in acute PUUV infection. ***Methods:*** The amount of dipstick albuminuria at hospital admission was analyzed in 205 patients with acute PUUV infection. Dipstick albuminuria at admission was graded into 3 categories: 0–1+, 2+, and 3+. In 70 patients, 24-h urinary excretion of protein, overnight urinary excretion of albumin, immunoglobulin (Ig) G, and α1-microglobulin also were measured over 3 consecutive days during the hospital stay. ***Results:*** Maximum median daily proteinuria, overnight albuminuria, and IgG excretion were observed over 5 days, while that of creatinine values was observed 9 days after the onset of the disease. The medians of maximum plasma creatinine levels during hospital stay were different in the 3 categories of dipstick albuminuria: 0–1+: 98 μmol/L (58-1,499), 2+: 139 μmol/L (71–829), and 3+: 363 μmol/L (51–1,285; $p < 0.001$). Dipstick albuminuria ≥2+ at admission could be detected in 89% of the patients who subsequently developed severe AKI. Glomerular proteinuria, but not tubular proteinuria (α1-microglobulin), correlated with the severity of the emerging AKI. ***Conclusion:*** In acute PUUV infection, maximum median proteinuria values preceded the most severe phase of AKI by a few days. A highly useful finding for clinical work was that a quick and simple albuminuria dipstick test at hospital admission predicted the severity of the upcoming AKI.
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Introduction

Hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) are the manifestations of hantavirus infections in humans [1–3]. HFRS is caused by Eurasian hantaviruses, whereas HCPS occurs in the America [1–3]. These syndromes have also been referred to as "hantavirus fever," as there are similarities in the clinical picture of the 2 syndromes [4, 5]. *Hantaan virus* causes HFRS in Asia, whereas *Puumala virus* (PUUV) and *Dobrava virus* (DOBV) are distributed in Europe [1–3]. PUUV, spread by bank voles (*Myodes glareolus*), causes most of the HFRS cases in Europe [1–3].

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PUUV infections are most common in Northern Europe, and high numbers of these infections are reported in Finland with 1,000–3,000 serologically diagnosed cases annually. It is also common in Sweden, Belgium, Germany, and Western Russia, but there are fewer cases in many countries of Southern Europe [6, 7].

The severity of hantavirus infection varies. HCPS can have a mortality of 35–50%, whereas HFRS caused by PUUV has a low mortality of 0.1% [2, 8]. The 3 typical features in all HFRS cases are renal involvement, thrombocytopenia, and increased capillary leakage [9–13]. In PUUV infection, the common clinical symptoms include sudden high fever with headache, abdominal and back pains, nausea, and visual disturbances, while serious hemorrhagic manifestations are rare [9–13]. The disease often leads to hospitalization and sometimes to intensive care unit (ICU) treatment, including renal replacement therapy (RRT) and mechanical ventilation [14].

Renal involvement of PUUV infection causes transient proteinuria, microscopic hematuria, and frequently acute kidney injury (AKI). Oliguria or anuria is followed by polyuria, and thereafter, spontaneous recovery takes place [9–13, 15]. RRT is needed in up to 6% of the hospitalized patients [10–13]. There is a genetic predisposition to the severity of PUUV infection [16, 17], and smokers develop a more severe AKI than non-smokers [18, 19]. The outcome of the AKI in PUUV infection is favorable, that is, without persisting sequelae [14].

The typical renal histopathologic finding in PUUV infection is acute tubulointerstitial nephritis, predominated by infiltrating lymphocytes, and also including plasma cells, monocytes, macrophages, and eosinophils [20, 21]. Proteinuria is detected in almost all patients, which is of the nephrotic range in up to one-third of them [9–11, 13, 22]. The pathogenesis of this massive proteinuria is unclear. In a recent retrospective study from Germany, urinary neutrophil gelatinase-associated lipocalin (NGAL) and urine albumin (U-alb) level predicted the severity of AKI in acute PUUV infection [23]. In this cohort, albuminuria was determined by urine albumin–creatinine ratio (ACR) in 24 patients and by dipstick in 59 patients, while tubular proteinuria was not addressed [23].

In the present study, our aim was to evaluate the prognostic significance of proteinuria on the severity of subsequent AKI in acute PUUV infection. Furthermore, we assessed the type and kinetics of proteinuria in relation to the course of PUUV infection.

Subjects and Methods

The cohort of this study consisted of 217 consecutive PUUV-infected patients treated at the Tampere University Hospital, Finland, during 1997–2014. The patients also participated in our previous studies [17, 22, 24–33]. All patients were examined during the acute phase of the disease, and the diagnosis of acute PUUV infection was serologically confirmed [34–37]. A detailed medical history was obtained and physical examination was performed. The median age was 41 (range 15–77) years, 12 patients (7%) were >65 years, and 138 patients (67%) were men. Patients <15 years of age were excluded. Also, 12 patients were excluded because of only one recorded creatinine value (5 patients) or missing urine dipstick test (7 patients). Altogether 205 patients were included in the analyses.

The following diagnoses before acute PUUV infection had been made in 56 (27%) patients: hypertension (n = 17), coronary artery disease (n = 7), rheumatoid arthritis (n = 5), diabetes (n = 4), bronchial asthma (n = 4), hypothyroidism (n = 4), celiac disease (n = 3), inflammatory bowel disease (n = 2), psychiatric disorder (n = 2), operated malignant disease (n = 2), cardiac conduction disorder (n = 2), aortic/mitral valve disease (n = 2), epilepsy (n = 2), osteoporosis (n = 2), sequelae of renal tuberculosis (n = 1), polycystic kidney disease (n = 1), operated meningioma (n = 1), chronic lymphatic leukemia in remission (n = 1), psoriasis (n = 1), prostate hyperplasia (n = 1), sleep apnea (n = 1), and transient ischemic attack (n = 1). Some patients had more than one disease, but none had known prior kidney insufficiency. Although 2 patients had a history of a kidney disease, both of them had normal kidney function before PUUV infection. None of the patients were given nonsteroidal anti-inflammatory drugs in the hospital, but possible use before hospitalization was not recorded.

Laboratory Determinations

In all 205 patients, U-alb dipstick test was performed at hospital admission, and daily plasma creatinine values during the hospital stay were determined (median number of determinations 5, range 2–15). The amount of albuminuria detected by dipstick test was graded into 3 categories: 0–1+ (n = 54), 2+ (n = 73), and 3+ (n = 78). In 70 consecutive patients treated during 1997–1999, analyses of 24-h urinary protein excretion, overnight excretion of albumin, immunoglobulin (Ig) G, and α1-microglobulin were performed during 3 consecutive days. The highest and lowest values of the variables measured during hospitalization for each patient were designated as the maximum and minimum values, respectively.

The diagnosis of PUUV infection in 1982–1989 was based on duplicate samples with 4-fold or greater rise in IgG titer by the immunofluorescence assay (IFA) [34]. Since 1989, recent PUUV infection was confirmed from a single serum sample by detecting the typical granular staining pattern in IFA [35] and/or low avidity of IgG antibodies to PUUV [36] and/or by detecting PUUV IgM antibodies by an "in-house" enzyme-linked immunosorbent assay based on a recombinant antigen [37]. The development and use of the above and other diagnostic methods have been described by Vaheri et al. [38].

Plasma creatinine was determined until 1999 by Vitros (Johnson & Johnson, Rochester, NY, USA) and thereafter by Cobas Integra (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Urine dipstick analysis was made by automated tests based on refractometry:

Table 1. The clinical and laboratory characteristics of 205 patients with acute Puumala hantavirus infection

Finding	Median	Range
Age, years	41	15–77
Duration of fever, days	6	2–19
Length of hospital stay, days	6	2–25
Body weight change during hospital stay, kg*	2.4	0–12
Hematocrit max	0.44	0.33–0.60
Platelets min, $\times 10^9$/L	61	3–249
Leukocytes max, $\times 10^9$/L	10.4	4.2–45.0
Plasma CRP max, mg/L	79	16–269
Plasma creatinine max, μmol/L	200	51–1,448
Plasma urea max (n = 110), mmol/L	17.7	2.1–52.8
Sodium min, mmol/L	132	109–142
Potassium max, mmol/L	4.3	3.3–5.5
Plasma albumin min (n = 113), g/L	27	11–39
Urinary protein excretion max (n = 70), g/24 h	1.80	0.14–17.78
Overnight urinary albumin excretion max (n = 70), μg/min	760	4–7,026
Overnight urinary IgG excretion max (n = 70), μg/min	173	3–1,565
Overnight urinary α1-microglobulin excretion max (n = 70), μg/min	28	2–209

* Difference between the highest and lowest weight during hospital stay; min, minimum; max, maximum; CRP, C-reactive protein; IgG, immunoglobulin G.

from 1997 using Miditron M (Roche), from 2004 Urisys 2400 or 1900 (Roche), and from 2009 until 2014 Siemens Clinitek Atlas or Advantus. The sensitivity of these tests to U-alb (1+) range is 0.15–0.3 g/L. The dipstick result 2+ stands for ≥1 g/L albumin and 3+ stands for ≥3 g/L albumin. The albumin dipstick tests do not react with urinary globulins or Ig light chains. Urine for the dipstick test was sampled on admission already at the emergency room.

In 70 patients, urine collection was started on the first evening of hospital care and continued for 3 days. The nightly collection period was from the time of the last voiding at bedtime until the last voiding on rising. The 24-h collection commenced immediately thereafter. The 24-h urinary protein excretion was measured by the pyrogallol red molybdate method (Olli C.; Kone Instruments, Helsinki, Finland) until 1998, then by using Cobas Integra (Roche Diagnostics) until 2008, and thereafter using Cobas 8000 analyzer (Roche Diagnostics). Timed overnight urinary excretions of albumin and α1-microglobubulin were measured using nephelometry (Behring Nephelometer II Analyzer, Behringwerke AG, Marburg, Germany).

Blood cell count was determined by hematological cell counters (Bayer Diagnostics, Elkhart, IN, USA), and sodium, potassium, urea, and albumin concentrations using routine automated chemistry analyzers. All laboratory determinations were performed by the laboratory Centre of the Pirkanmaa Hospital District (later named Fimlab Laboratories), Tampere, Finland.

Statistical Analyses

Medians and ranges were given for skewed continuous variables and numbers and percentages for categorical variables. Spearman rank correlations were calculated. Categorical data were analyzed by the χ^2 test or the Fisher exact test, and groups were compared using the Mann–Whitney U test or the Kruskal–Wallis test, as appropriate. All tests were 2-sided, and the analyses were performed using SPSS (version 20) statistical software (IBM, Chicago, IL, USA).

Results

The clinical and laboratory data of the 205 patients are shown in Table 1. The median interval between the onset of symptoms (i.e., fever) and admission to hospital was 4 (range 1–15) days. Median duration of the hospital stay was 6 (range 2–25) days. Plasma creatinine was elevated (>100 μmol/L) in 153 (75%) patients. Six patients (3%) were in clinical shock at admission. Nine patients (4%) needed RRT, and among them, the median creatinine concentration was 706 μmol/L (range 265–1,285). Among those treated with RRT, 3 subjects were in clinical shock and they had the lowest creatinine values (265–473 μmol/L) in this group of patients. After hospitalization 2 of these subjects were immediately transferred to the ICU for RRT due to oliguria or anuria, fluid retention, and hypotension. None of the 205 patients had a urinary tract infection and all recovered.

In 70 consecutive patients, 24-h urinary protein excretion, overnight excretion of albumin, α1-microglobulin, and IgG were measured over 3 consecutive days during the hospital stay. The maximum 24-h urinary protein ex-

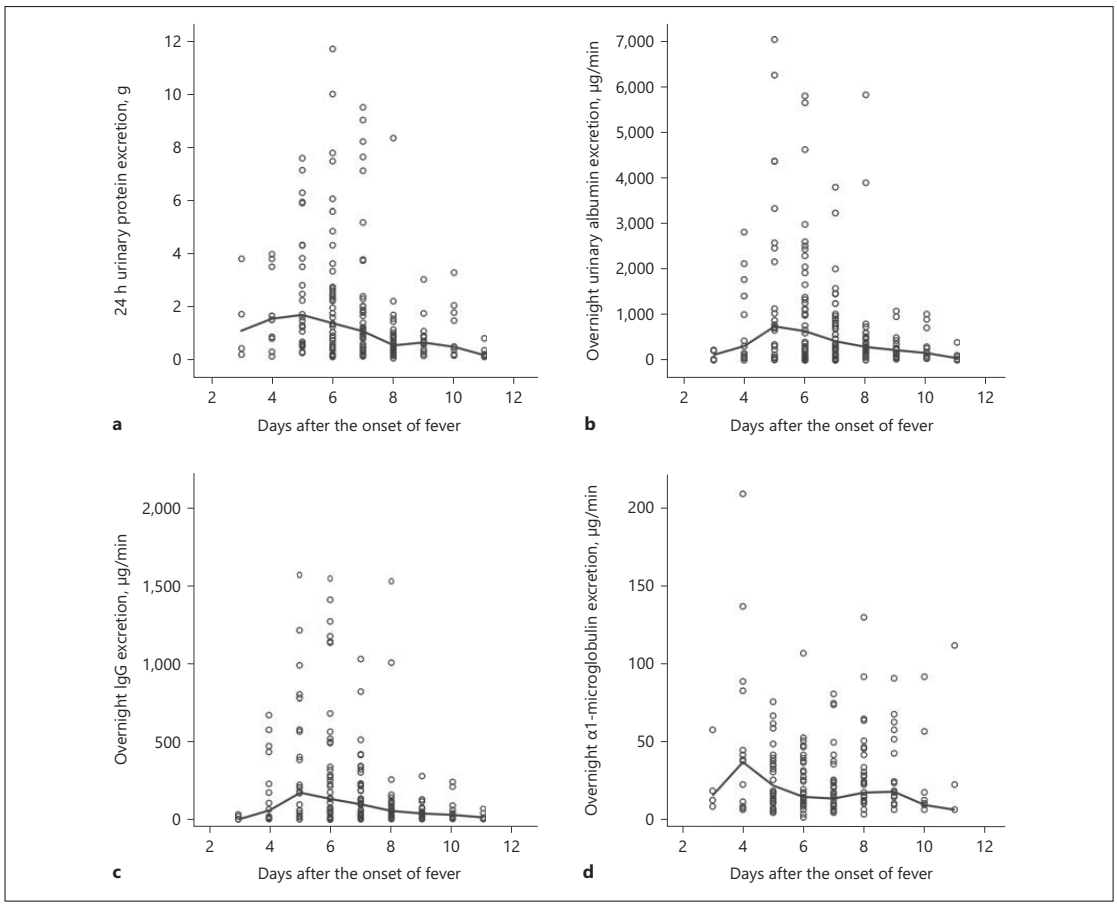

Fig. 1. Scatter plots show 24-h urinary excretion of protein (**a**), overnight urinary excretion of albumin (**b**), immunoglobulin (Ig) G (**c**), and α1-microglobulin (**d**) in relation to the onset of fever in 70 PUUV-infected patients; **a–d** depict values on 3 consecutive days in each patient. Every circle represents one measurement, the line depicts median values, outliers and extremes are omitted.

cretion ranged from 0.14 to 17.78 g/24 h and was of nephrotic range (>3.5 g/24 h) in 34% of patients. The highest 24-h urinary protein excretion (peak median 1.67 g/24 h, range 0.26–17.78) was detected on the fifth day after the onset of fever (Fig. 1a). Glomerular proteinuria, that is, overnight excretion of albumin (peak median 734 µg/min, range 12–7,026; Fig. 1b) and overnight excretion of IgG (peak median 183 µg/min, range 6–1,565; Fig. 1c) peaked on the fifth day as well. Tubular proteinuria, that is, increased overnight urinary excretion of α1-microglobulin (>7 µg/min), was detected in 90% of the patients. The excretion was highest (peak median 38 µg/min, range 7–209) on the fourth day after the onset of fever (Fig. 1d). Plasma creatinine value (peak median 229 µmol/L, range 68–725) was highest on the ninth day after the onset of fever (Fig. 2).

The maximum 24-h urinary protein excretion correlated slightly with maximum plasma creatinine level (r = 0.30, p = 0.012), while there was a higher correlation between glomerular proteinuria (maximum overnight excretion of albumin and IgG) and maximum plasma creatinine level (r = 0.41; p < 0.001 and r = 0.44; p < 0.001, respectively). Tubular proteinuria (maximum overnight α1-microglobulin excretion) did not correlate with max-

imum plasma creatinine concentration (r = 0.13; p = 0.291). Maximum overnight α1-microglobulin excretion correlated with maximum C-reactive protein (CRP) level (r = 0.39; p = 0.001), but not with other variables reflecting disease severity, that is, length of hospital stay, weight change during hospital stay, maximum hematocrit, minimum platelet count, or maximum blood leukocyte count (data not shown).

Table 2 shows the clinical and laboratory data of 205 patients according to the 3 groups of U-alb dipstick test result at hospital admission. Patients with higher albuminuria by dipstick test had greater change in body weight during hospitalization, higher maximum hematocrit, lower minimum sodium concentration, and lower minimum plasma albumin than patients with less or no albuminuria. The results of these findings reflect the degree of capillary leakage. They also had more severe AKI (higher maximum plasma creatinine, urea, and potassium concentrations) and longer hospital stay, reflecting the severity of the disease. Higher albuminuria by dipstick at admission was associated with higher overnight urinary excretion of albumin and IgG, but not of α1-microglobulin. All the above associations were statistically significant (for p values see Table 2). Platelet count was decreased (≤150 × 10^9/L) in 197 patients (96%), but the minimum platelet count did not differ between the groups. The groups showed differences in the concentration of CRP, but the level of CRP was not associated with the amount of dipstick albuminuria. Higher category of dipstick albuminuria was numerically related to higher 24-h urinary protein excretion and lower plasma albumin, but these differences were not statistically significant. Among the 9 patients needing RRT, the dipstick albuminuria categories were 3+ in 6 subjects, 2+ in 1 subject, and 0–1+ in those 2 patients who were initially in clinical shock and treated at the ICU.

To evaluate whether the duration of symptoms before admission to the hospital influenced the ability of U-alb dipstick test to predict the severity of AKI, we divided the patients into 2 subgroups: admission on days 1–4 (128 patients) or on day 5 or later (77 patients) after the onset of fever. In both groups, the amount of dipstick albuminuria at admission was associated with median maximum plasma creatinine level: (1) admission on days 1–4: U-alb 0/1+: 96 μmol/L (range 52–1,499), U-alb 2+: 123 μmol/L (range 71–749), U-alb 3+: 354 μmol/L (range 51–1,285; p < 0.001); (2) admission on days ≥5: U-alb 0/1+: 118 μmol/L (range 58–874), U-alb 2+: 199 μmol/L (range 76–829), U-alb 3+: 376 μmol/L (range 93–1,153; p < 0.001).

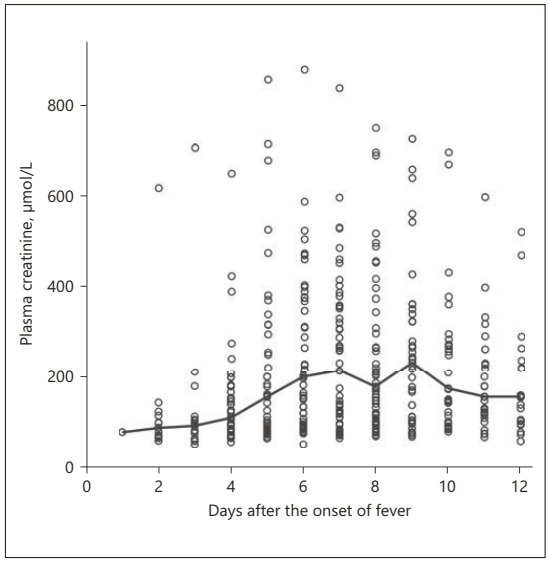

Fig. 2. Scatter plot shows plasma creatinine values in relation to the onset of fever in 70 PUUV-infected patients during hospital stay. Every circle represents one measurement, the line depicts median values.

Severe stage 3 AKI (KDIGO, plasma creatinine ≥353.6 μmol/L [39]) was discovered in 63 (31%) patients during the hospitalization. Albumin dipstick test ≥2+ at admission could detect 89% of those who subsequently developed severe AKI (creatinine ≥353.6 μmol/L). Furthermore, albumin dipstick test 3+ at admission showed high positive predictive value of 82% for maximum plasma creatinine >200 μmol/L (median in the study population) with a negative predictive value of 69%.

Discussion

The present study showed that in acute PUUV infection, maximum proteinuria preceded the most severe phase of AKI by some days. Glomerular proteinuria, but not tubular proteinuria, correlated with the severity of AKI. To our knowledge, this finding has not been previously reported in HFRS. The amount of albuminuria detected by dipstick test at hospital admission predicted the severity of AKI in PUUV infection. It was also associated with several other disease severity markers, many of which reflect the general capillary leakage typical of HFRS.

Table 2. Clinical and laboratory data of 205 patients divided into 3 groups according to urine dipstick protein category at hospital admission

	U-alb 0/1+	U-alb 2+	U-alb 3+	*p* value
Number of patients	54	73	78	
Age, years	41 (22–77)	43 (22–74)	40 (15–65)	0.294
Length of hospital stay, days	5 (3–22)	6 (3–15)	7 (2–14)	0.026
Body weight change during hospital stay, kg*	1.6 (0–10.8)	2.2 (0–10.0)	3.7 (0–12.0)	<0.001
Hematocrit max	0.42 (0.33–0.59)	0.44 (0.34–0.59)	0.46 (0.34–0.60)	0.001
Platelets min, × 10^9/L	68 (3–249)	61 (15–198)	56 (5–187)	0.232
Leukocytes max, × 10^9/L	9.2 (5.1–38.6)	9.0 (4.2–31.2)	13.0 (5.7–45.0)	<0.001
Plasma CRP max, mg/L	74 (16–236)	92 (20–269)	68 (21–214)	0.012
Plasma creatinine max, μmol/L	98 (52–1,447)	139 (71–829)	363 (51–1,285)	<0.001
Plasma urea max, mmol/L (*n* = 110)	9.9 (2.0–52.4)	13.3 (3.9–39.7)	25.7 (2.7–52.8)	<0.001
Sodium min, mmol/L	135 (109–141)	133 (120–141)	130 (113–142)	0.001
Potassium max, mmol/L	4.1 (3.3–5.2)	4.2 (3.3–5.3)	4.4 (3.3–5.5)	0.001
Plasma albumin min, g/L (*n* = 113)	30 (11–39)	28 (21–37)	27 (18–37)	0.161
Urinary protein excretion max, g/24 h (*n* = 70)	0.57 (0.14–9.50)	1.74 (0.18–17.78)	2.22 (0.30–10.00)	0.076
Overnight urinary albumin excretion max, μg/min (*n* = 70)	104 (4–4,617)	756 (12–6,246)	1,016 (136–7,026)	0.007
Overnight urinary IgG excretion max, μg/min (*n* = 70)	16 (7–1,267)	211 (3–1,565)	226 (25–1,542)	0.005
Overnight urinary α1-micro-globulin excretion max, μg/min (*n* = 70)	20 (10–89)	38 (9–209)	30 (2–130)	0.693

Values are expressed as medians (range).
* Difference between the highest and lowest weight during hospital stay; min, minimum; max, maximum; CRP, C-reactive protein.

In previous times, when reliable serological testing was not yet readily available to diagnose acute PUUV infection, kidney biopsies were performed to verify the reason of AKI in PUUV-infected patients. These biopsies have shown that the characteristic histological finding of acute PUUV infection is acute tubulointerstitial nephritis [20, 21]. Although intense proteinuria is not typical for tubulointerstitial nephritis, proteinuria in acute PUUV infection exceeds the nephrotic range in one-third of the patients [9–11, 13, 22], a finding that well corresponds with the present results. The contribution of tubular injury to the proteinuria in PUUV-infected patients is exemplified by the loss of low-molecular-weight proteins in the urine [40, 41]. As kidney biopsies have usually been performed after the acute-phase thrombocytopenia has been resolved to avoid biopsy-related bleeding complications, the timing of the biopsies has probably influenced the renal histological findings of the disease.

The glomerular alterations of acute PUUV infection in light microscopy are minor [20], but the rapid onset of proteinuria suggests that alterations have taken place in vascular barrier function. The non-selective nature of the proteinuria indicates that the glomerular barrier is defective in acute PUUV infection [40, 41]. However, even in the case of massive transient proteinuria, the associated glomerular lesions with respect to adhesion molecules, cytokines, and cell infiltration remain negligible [21]. The hantaviruses can infect not only vascular endothelial cells, but also glomerular endothelial cells, tubular cells, and podocytes, disrupting the cell-to-cell contacts in all of these cell types [42]. Indeed, in electron microscopic studies, fusion of podocyte foot processes was already found in 1978 [43, 44]. Recently, 2 reports confirmed severe ultrastructural changes in podocytes with foot process effacement in 3 patients biopsied in the acute phase of hantavirus infection [45, 46]. It seems plausible that podocyte dysfunction and disruptions of the cell-to-cell contacts impair the barrier function and result in intense glomerular proteinuria in the early phase of PUUV infection. In this study, glomerular proteinuria reached the highest values on the fifth day after the first clinical symptoms of the disease, and thereafter, urine protein excretion decreased, suggesting that proteinuria in this disease is rapidly resolving. However, we do not have data about urine protein excretion after hospitalization, and the disappearance of proteinuria in PUUV-infected patients is a subject for further studies.

In the present study, many variables reflecting capillary leakage were associated with the amount of dipstick albuminuria at hospital admission (Table 2). Thus, glomerular proteinuria may reflect the increased capillary

leakage in acute PUUV infection. The main hantavirus targets are the endothelial cells of the post-capillary venules in various organs [3, 12], while increased vascular permeability is a typical finding in all hantavirus infections [47]. The pathogenesis of this has not been fully elucidated, but it may involve the release of bradykinin, which increases vascular permeability in various pathological conditions. Indeed, the selective bradykinin type 2 receptor antagonist, icatibant, was successfully administered in 2 cases of life-threatening PUUV infection [48, 49]. In imaging studies of PUUV-infected patients, signs of fluid accumulation and edema have been frequently found [50]. We previously reported that during the acute phase of PUUV infection, both renal parenchymal swelling and high arterial resistive index in renal ultrasound were associated with the severity of AKI [51, 52]. The amount of urinary 24-h protein excretion, however, was not associated with quantitative or qualitative findings in the renal ultrasound study [51, 52]. In addition to capillary leakage, possible explanations to kidney swelling in acute PUUV infection are tubulointerstitial inflammation and medullary hemorrhages [20, 21, 46].

We found that maximum CRP level was not associated with the amount of albuminuria detected by the dipstick test. Inflammatory response may nevertheless have a major role in the pathogenesis of proteinuria and plasma leakage in HFRS. Maximum urinary protein excretion was found to correlate with plasma and urine interleukin-6 concentrations but not with complement activation in the acute phase of PUUV infection [22, 27, 53].

Several mechanisms can lead to AKI during acute PUUV infection. In 17 French patients, acute tubular necrosis (ATN) was the major histologic feature in 88% of cases, along with microvascular inflammation in peritubular capillaries [46]. Viral stimulus can trigger a major inflammatory response, causing tissue damage and ATN [54]. In kidney epithelial cell lines, apoptosis was reported in response to hantavirus infection [55, 56]. Additional mechanisms of ATN in PUUV-infected patients include hemodynamic alterations and intravascular hypovolemia, secondary to the capillary leakage. The use of non-steroidal anti-inflammatory drugs is another putative cause for ATN, and may contribute to the severity of AKI in PUUV infection [57]. These drugs were not given to the present patients at hospital, but possible usage before hospital admission was not recorded.

In chronic glomerular diseases, non-selective proteinuria has been associated with tubular damage and progression of renal failure [58], but the role of glomerular proteinuria in the pathogenesis of AKI is unclear. Preexisting proteinuria is a risk factor for AKI in various clinical situations [59]. Only few studies have examined the significance of de novo proteinuria during AKI. New-onset urinary dipstick proteinuria was associated with severe AKI in critically ill septic patients [60] and increased risk of developing AKI in patients with severe burns [61]. In cardiac surgery patients, high amount of postoperative dipstick proteinuria was associated with the risk of AKI [62]. In our study, glomerular proteinuria correlated with the severity of upcoming AKI, but tubular proteinuria did not. Furthermore, the peak excretion of albumin and IgG preceded the most severe phase of AKI by 4 days. Thus, alterations in glomerular permeability reflect the severity of upcoming AKI in acute PUUV infection. As a limitation of the present study, this type of proteinuria could only be evaluated in one-third (70 of 205) of the patients.

In a recent study, aimed at identifying patients who are at lower risk of developing severe AKI during acute PUUV infection, albuminuria defined by a urine ACR >0.25 g/g was one of the 3 risk factors that predicted the risk of AKI [63]. The other 2 factors were thrombocytopenia and the level of CRP [63]. Urinary NGAL was also found to predict the severity of AKI in PUUV infection [23]. When compared with urine ACR and NGAL determinations, albumin dipstick test is quick, inexpensive, and readily available. As albumin dipstick test result ≥2+ at hospital admission identified 89% of the patients who subsequently developed severe AKI, this simple test is a clinically applicable tool for the evaluation of severe AKI risk and the need for hospital treatment. Of note, the preceding duration of the symptoms of PUUV infection did not influence the ability of U-alb dipstick test to predict the severity of upcoming AKI.

In conclusion, maximum proteinuria preceded the most severe phase of AKI by some days in acute PUUV infection. Glomerular proteinuria, but not tubular proteinuria, correlated with the severity of the emerging AKI. The influence of proteinuria on the mechanisms of AKI during hantavirus infections is an interesting subject for further investigations. Finally, the determination of albuminuria by urine dipstick test at hospital admission predicted the severity of upcoming AKI in acute PUUV infection. It also associated with several variables that reflect increased capillary leakage. The predictability of this simple test should be evaluated in more severe HFRS cases caused by HTVN in Asia or DOBV in Europe, and in patients with HCPS in the Americas.

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Statement of Ethics

All patients provided a written informed consent and the study was approved by the Ethics Committee of Tampere University Hospital.

Disclosure Statement

The authors have no conflicts of interest to declare. We certify that the submission is original work and is not being considered for publication elsewhere, in whole or in part, except in abstract form.

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PUBLICATION II

Hematuria is a marker for the severity of acute kidney injury but does not associate with thrombocytopenia in acute Puumala hantavirus infection

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Hematuria is a marker for the severity of acute kidney injury but does not associate with thrombocytopenia in acute Puumala hantavirus infection

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Abstract

Backgound: Puumala hantavirus (PUUV) causes hemorrhagic fever with renal syndrome characterized by thrombocytopenia, capillary leakage and acute kidney injury (AKI) with proteinuria and hematuria. Although the typical histologic lesion is acute tubulointerstitial nephritis, the amount of glomerular proteinuria predicts the severity of upcoming AKI. Here we studied the associations of hematuria and proteinuria with the severity of emerging AKI, thrombocytopenia, and markers of coagulation and fibrinolysis in PUUV infection.

Methods: We examined 205 consecutive patients treated for serologically confirmed acute PUUV infection at Tampere University Hospital during 1997-2014. The patients were divided into three groups according to the combined positive result in urine hemoglobin and albumin dipstick tests: 0-2+ (n=58), 3-4+ (n=100), and 5-6+ (n=47).

Results: The medians of maximum creatinine concentrations in the three groups were: 0-2+ 100 μmol/L (range 52-1499), 3-4+ 204 μmol/L (range 65-1071), and 5-6+ 361 μmol/l (range 51-1285) ($p<0.001$). The number of blood platelets ($p=0.069$), and the levels of fibrinogen, prothrombin fragments F1+2, and d-dimer ($p=0.602$, $p=0.113$, $p=0.289$, respectively) were not significantly different between the groups. When the amount of hematuria in the dipstick test was examined separately, no association with thrombocytopenia was detected ($p=0.307$ between groups 0, 1+, and 2-3+).

Conclusions: Combined positive result of hematuria and proteinuria in the dipstick test at hospital admission predicted the severity of upcoming AKI in acute PUUV infection. As hematuria was not associated with the severity of thrombocytopenia, it did not indicate increased bleeding tendency, but was rather a marker of acute kidney injury.

Introduction

Hantaviruses infect many species of rodents, shrews, moles, and bats and, in humans, cause hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas [1]. Puumala virus (PUUV), carried by the bank vole, causes a HFRS called nephropathia epidemica (NE) [1]. Most of the European HFRS cases are caused by PUUV and the majority of all NE cases are reported in Finland, where thousands of serological diagnoses are made annually [2].

The typical features in all HFRS cases are fever, increased vascular permeability, renal involvement, and thrombocytopenia [3, 4]. Renal involvement in PUUV infection causes temporarily decreased glomerular filtration, transient, often massive, proteinuria, hematuria, and oliguria, which is followed by polyuria and spontaneous recovery [3-5]. Smokers have a more severe acute kidney injury (AKI) than non-smokers [6, 7]. However, the outcome of AKI in PUUV infection is favorable [8].

Despite often substantial thrombocytopenia, serious hemorrhages are rare, while mild bleeding manifestations, such as conjunctival bleeding, epistaxis, or petecchiae, occur in about one-third of the patients [3]. The mortality of PUUV infection is low, ranging from less than 0.1% in Finland to 0.4% in Sweden [9, 10]. However, the disease often leads to hospitalization and intensive care unit treatment, including renal replacement therapy, may be needed [3].

The characteristic histopathologic renal finding in PUUV infection is acute tubulointerstitial nephritis, and the infiltrating cells include lymphocytes, plasma cells, monocytes, macrophages, and polymorphonuclear cells [11, 12]. Glomerular changes are mild and do not correlate with the amount of proteinuria [11, 12]. Medullary hemorrhages have been found in 20% of renal biopsy samples and almost all medulla-containing biopsy specimens have revealed hemorrhages [11-13].

Microscopic hematuria is present in a vast majority (58-94%) of patients infected by PUUV [5, 11, 12, 14]. Recently, albuminuria was found to predict the severity of upcoming AKI [15]. However, the significance of hematuria in PUUV infection has not been established. In this study, we examined the associations of hematuria, and combined hematuria and albuminuria, with emerging AKI, thrombocytopenia, and markers of coagulation and fibrinolysis in acute PUUV hantavirus induced HFRS.

Materials and Methods

Subjects

The study cohort consisted of 217 consecutive patients with acute PUUV infection, who also participated in our previous studies [6, 8, 14-16]. The patients were treated at the Tampere University Hospital, Finland, during 1997-2014. All patients were examined during the acute phase of the disease, and the diagnosis of acute PUUV infection was serologically confirmed in all of them [17-19]. A detailed past and current medical history was obtained and careful physical examination performed. The

median patient age was 41 (range 15-77) years, 12 patients (7%) were older than 65 years, and 138 patients (67%) were males. Patients younger than 15 years of age were excluded from the study as well as patients with a missing dipstick test. Altogether 205 patients were included in the analyses.

Fifty-six patients (27%) had one or more diseases diagnosed before PUUV infection. The diseases have been described in detail earlier [15]. None of the patients had any known chronic kidney disease prior to PUUV infection. One patient had suffered from renal tuberculosis and another patient had polycystic kidney disease, but they did not have chronic impairment of kidney function. All patients provided a written informed consent and the study was approved by the Ethics Committee of Tampere University Hospital.

Laboratory determinations

In all 205 patients, urine dipstick test was performed at hospital admission, and daily plasma creatinine and platelet values during the hospital stay were determined. The amount of hematuria detected by dipstick test was graded into three categories: 0 (28 patients), 1+ (94 patients), and 2-3+ (83 patients). Urine sediment was also assessed in 189 patients, either by microscopy or by flow cytometry and coefficient factor 1 erythrocyte/high power field = 5.8 x 10^6/L was used to standardize the results.

Recent PUUV infection was confirmed from a single serum sample by detecting the typical granular staining pattern in immunofluorescence assay [17], and/or low avidity of IgG antibodies to PUUV [19], and/or by detecting PUUV IgM antibodies by an 'in-house' enzyme-linked immunosorbent assay (ELISA) based on a recombinant antigen [18].

Plasma creatinine was determined until 1999 by Vitros (Johnson & Johnson, Rochester, N.Y., USA) and thereafter by Cobas Integra (F. Hoffman- La Roche Ltd., Basel, Switzerland). Urine dipstick analysis was made by automated tests based on refractometry: From 1997 using Miditron M (Roche), from 2004 Urisys 2400 or 1900 (Roche), and from 2009 until 2014 Siemens Clinitec Atlas or Advantus. Urine for the dipstick test was sampled on admission already at the emergency room.

Blood cell count was determined by hematological cell counters (Bayer Diagnostics, Elkhart, IN, USA). In 43 patients, plasma fibrinogen levels were assessed from citrate-anticoagulated samples after centrifugation at 2000 g for 10 min at room temperature using a viscosity-based detection system (Diagnostica Stago; reference range 2.0–4.0 g/l). D-dimer (Tina-quant D-Dimer immunoturbidimetric assay, Roche Diagnostics, Mannheim, Germany) and prothrombin fragments (F1+2, a monoclonal enzyme immunoassay Enzygnost F1+2, Siemens Healthcare Diagnostics) were also assessed in the same 43 patients.

In 70 patients, urine collection was started on the first evening of hospital care and continued for 3 days. The nightly collection period was from the time of the last voiding at bedtime until the last voiding on rising. The 24-hour collection commenced immediately thereafter. The 24-hour urinary protein excretion was measured by the pyrogallol red molybdate method (Olli C.; Kone Instruments, Helsinki, Finland) until 1998, then by using Cobas Integra (Roche diagnostics) until 2008, and thereafter

using Cobas 8000 analyzer (Roche diagnostics). Timed overnight urinary excretion of albumin and α1-microglobubulin was measured using nephelometry (Behring Nephelometer II Analyzer, Behringwerke AG, Marburg, Germany).

Other analytical procedures were performed using routine automated chemistry analyzers. The highest and lowest values of the variables measured during hospitalization for each patient were designated as the maximum and minimum values, respectively. All laboratory determinations were performed by the laboratory Centre of the Pirkanmaa Hospital District (later named Fimlab Laboratories), Tampere, Finland.

Statistical analyses

Medians and ranges were given for skewed continuous variables and numbers and percentages for categorical variables. Spearman's rank correlations were calculated. Categorical data were analyzed using the x^2 test or the Fisher's exact test, and groups were compared using the Mann-Whitney *U*-test or the Kruskal-Wallis test, as appropriate. All tests were two-sided, and the analyses were performed using SPSS (version 20) statistical software (IBM, Chicago, IL).

Results

The clinical characteristics and laboratory findings of the patients have been described earlier [15]. The patients were admitted to the hospital median 4 (range 1-15) days after the onset of fever. Median duration of the hospital stay was 6 (range 2-25) days. Six patients (3%) were in clinical shock at admission, while 9 patients (4%) needed renal replacement therapy during hospitalization. All patients recovered.

Altogether 177 patients (86%) had hematuria detected by dipstick test (1-3+) at admission. In 94 patients (46%) hematuria was mild, categorized as 1+, while 83 patients (40%) had grade 2-3+ hematuria. In 189 patients (92%) also urine sediment was examined. The amount of erythrocytes in the sediment was elevated (>2/high power field) in 122 (65%) patients.

The degree of hematuria assessed by dipstick associated with the severity of AKI. Figure 1 shows the association of the degree of dipstick-verified hematuria with maximum plasma creatinine level. Also, the amount of erythrocytes in the sediment correlated slightly with the severity of AKI (r=0.168 for correlation with maximum plasma creatinine, p=0.021). The associations of dipstick-verified hematuria with various clinical and laboratory variables are shown in Table I. Patients with more pronounced hematuria had greater change in weight during hospitalization (reflects fluid retention during the oliguric phase), lower minimum urinary output, and higher maximum plasma creatinine and urea concentrations. Patients with higher degree of hematuria also had lower minimum albumin and sodium concentrations, and lower minimum hematocrit. The inflammatory variables C-reactive protein (CRP) and leukocyte count did not associate with the amount of dipstick-verified hematuria. Higher dipstick-verified

hematuria associated with greater urinary excretions of 24-hour protein and overnight albumin, but not with that of overnight urinary α1-microglobulin (i.e. tubular proteinuria) (Table I).

There was no association of dipstick-verified hematuria with thrombocytopenia. The coagulation and fibrinolysis markers fibrinogen, prothrombin fragments F1+2, and d-dimer had no association with the amount of hematuria assessed by dipstick (Table I) or erythrocytes in the urine sediment (data not shown). Minimum blood platelet count did not associate with the amount of erythrocytes in the sediment ($r=-0.124$, $p=0.088$). The amount of hematuria measured by dipstick test associated with the amount of erythrocytes in urine sediment (Table I).

The amount of dipstick-verified hematuria (grading 0-3+) was associated with the amount of dipstick-verified albuminuria (grading 0-3+) ($p<0.001$). Out of the patients, 57% with grade 3+ hematuria had also grade 3+ albuminuria, whereas none of the patients without dipstick-verified hematuria (grade 0) had grade 3+ albuminuria. The amount of dipstick-verified hematuria did not associate with the amount of dipstick-verified leukocyturia ($p=0.749$).

To further examine the associations of dipstick-verified hematuria with the severity of AKI, we calculated the influence of combined positive results in the dipstick hematuria and albuminuria tests (range from 0 to 3+ in each). With every positive semiquantitative step further (combined range outcome from 0 to 6+) in these tests, the plasma creatinine level rose: 0 creatinine 94 µmol/L (range 58-874), 1+ creatinine 101 µmol/L (range 58-1499), 2+ creatinine 102 µmol/L (range 52-541), 3+ creatinine 138 µmol/L (range 65-841), 4+ creatinine 256 µmol/L (range 69-1071), 5+ creatinine 361 µmol/L (range 51-1285), 6+ creatinine 426 µmol/L (range 160-1156) ($p<0.001$). Thereafter, we divided the patients into three groups according to the combined results of these two dipstick tests: 0-2+ (58 patients), 3-4+ (100 patients), and 5-6+ (47 patients) (Table II). Higher number of positive results in these two tests was associated with more severe upcoming AKI, and the patients stayed longer at the hospital. Overnight urinary albumin excretion also associated with higher number of positive results in the dipstick tests. Blood platelet level or the coagulation and fibrinolysis markers did not associate with the combined number of positive dipstick test results.

Discussion

To our knowledge this is the first study in which hematuria was systematically examined with respect to the severity of the emerging AKI and glomerular and tubular proteinuria during hantavirus infection, and the possible relation to laboratory findings reflecting hemostasis was also addressed. The present study showed that hematuria in acute PUUV hantavirus infection was associated with the severity of AKI, but not with thrombocytopenia or markers of coagulation and fibrinolysis. Furthermore, this study pointed out that when evaluating the severity of the developing AKI, both dipstick-verified hematuria and albuminuria should be considered.

Hematuria was detected by dipstick test in 86% of the 205 patients in the present study. This is well in line with previous findings, where microscopic hematuria was found in the majority, 58-94%, and gross hematuria in 3% of patients with PUUV infection [3, 5, 11, 14]. Altogether, hematuria is far more common in NE than in other types of acute tubulointerstitial nephritis, in which only about one-third of the patients present with hematuria [20].

We found that the degree of hematuria detected by dipstick test was associated with the severity of the upcoming AKI. The degree of dipstick-verified hematuria also associated with albuminuria measured by dipstick, 24-hour urine protein excretion, and overnight albumin excretion. Dipstick-verified hematuria did not associate with overnight α1-microglobulin excretion. Recently, we found that glomerular proteinuria, but not tubular proteinuria (α1-microglobulin), predicted the severity of AKI in NE [15]. More pronounced dipstick-verified hematuria was related to greater change in body weight, as well as lower minimum hematocrit, albumin, and sodium levels. All of these variables probably reflect fluid retention during the oliguric phase of PUUV infection, and they can be considered as consequences of more severe AKI.

Previously, a slight but significant correlation between the degree of microscopic hematuria and maximum creatinine level in PUUV infection was reported, in agreement with the present results [12]. Hematuria has also been associated with the development of polyuria, a typical sign of renal involvement in PUUV infection [21]. Furthermore, in HFRS caused by Hantaan virus, the presence of microscopic hematuria associates with the risk for developing oliguric AKI [22]. On the other hand, in a study aimed at identifying patients who were at lower risk of developing severe AKI, hematuria was not found to be a risk factor for severe AKI when compared with mild to moderate AKI [23]. However, the research approach was different from our study and the number of patients was lower, which may explain the divergent results.

In the present study, the amount of hematuria verified by dipstick test was not associated with inflammatory markers, i.e. blood leukocyte and CRP levels. Moreover, hematuria did not associate with the levels of blood platelets or markers of coagulation and fibrinolysis. In concordance with the present results, a previous German study did not find a difference in the presence of hematuria between patients with severe and non-severe thrombocytopenia [24]. PUUV infection is a HFRS with mild bleeding tendency. However, some bleeding manifestations, e.g. conjunctival bleeding, epistaxis, or petecchiae, occur in about one-third of the patients [3]. There are also case reports of serious bleedings, such as hypophyseal hemorrhages [25], while medullary hemorrhages are common findings in renal biopsy specimens [12]. In theory, hematuria could well be a sign of bleeding tendency in this HFRS, but our findings suggest that this is not the case. In our previous study, thrombocytopenia was not found to associate with AKI [16]. This is in line with the findings of the present study, where hematuria associated with AKI, but not with thrombocytopenia.

Previously, the amount of hematuria has not been found to be related to the extent of renal histologic findings [12]. It cannot be, however, excluded that medullary hemorrhages contribute to the

occurrence of hematuria during PUUV infection. When evaluating previous results regarding the relationship between the amount of hematuria and findings in renal biopsies, it must be taken into account that kidney biopsies have usually been performed after the acute-phase thrombocytopenia has resolved to avoid biopsy-related bleeding complications. Urine samples, on the contrary, have typically been collected already on admission to the hospital. Thus, the timing of the biopsies may have influenced the results.

The amount of erythrocytes in urine sediment was associated with dipstick-verified hematuria. Urine sediment erythrocyte count also correlated with the severity of AKI, but not with platelet count or the levels of the markers of coagulation and fibrinolysis. Thus, hematuria measured using this method provided corresponding results to the semi-quantitative dipstick hematuria test.

The significance of combined positive results for dipstick-verified hematuria and albuminuria was also analyzed. Higher number of positive results in these two tests was associated with higher plasma concentration of maximum creatinine. The minimum hematocrit, albumin, and sodium concentrations were, in turn, lower with higher number of positive dipstick tests, probably reflecting fluid retention and the severity of AKI. When evaluating the significance of hematuria and albuminuria together, the predictive value for the upcoming AKI was even better than that of hematuria alone. Blood platelet count or the levels of coagulation and fibrinolysis markers did not associate with the number of positive dipstick test results, and also failed to predict the severity of AKI in this patient population.

In conclusion, the number of combined positive results in urine hemoglobin and albumin dipstick tests at hospital admission predicted the severity of the upcoming AKI during acute PUUV hantavirus infection. Hematuria alone was associated with the severity of AKI, but not with the severity of the emerging thrombocytopenia. This suggests that hematuria did not indicate higher bleeding tendency, but was rather a marker of kidney injury during acute hantavirus infection.

Table I Clinical and laboratory findings in 205 patients with Puumala hantavirus infection divided into three groups according to urine hemoglobin dipstick category.

	U-Eryt 0 **n=28**	**U-Eryt 1+** **n=94**	**U-Eryt 2-3+** **n=83**	**p-value**
Hospital stay (days)	5.5 (3-22)	6.0 (3-25)	6.0 (2-16)	0.181
Change in body weight (kg)	1.5 (0-9.4)	2.1 (0-10.7)	2.8 (0-12.0)	0.015
Urinary output min (ml/day)	1640 (0-5720)	1540 (100-4920)	1060 (50-7000)	0.034
Laboratory findings in plasma and blood				
P-Creatinine max (µmol/L)	99 (58-874)	175 (52-1499)	265 (51-1285)	0.001
P-Urea max (mmol/L) (n=110)	7.7 (2.7-20.3)	17.7 (2.1-52.4)	22.1 (2.7-52.8)	0.002
P-Alb min (g/L)	32 (11-37)	29 (20-39)	26 (18-39)	0.002
P-Sodium min (mmol/L)	135 (126-139)	132 (114-142)	131 (109-141)	0.005
Hematocrit min	0.38 (0.25-0.43)	0.36 (0.25-0.43)	0.35 (0.22-0.46)	0.022
Leukocytes max ($x10^9$/L)	9.8 (4.2-38.6)	10.7 (5.4-31.2)	10.4 (4.4-45.0)	0.443
P-C-reactive protein max (mg/L)	74 (16-236)	75 (20-244)	84 (16-269)	0.436
Laboratory findings in urine				
dU-Protein max (g/day) (n=70)	0.7 (0.3-3.7)	2.6 (0.3-10.0)	1.8 (0.1-17.8)	0.019
cU-Albumin max (µg/min) (n=70)	97 (14-738)	987 (14-7026)	738 (4-6246)	0.005
cU-α1-microglobulin max (µg/min) (n=70)	42 (20-89)	21 (7-130)	27 (7-209)	0.280
U-Erythrocytes (per high power field)	2 (1-132)	3 (0-41)	8 (0-401)	<0.001
Laboratory findings reflecting hemostasis				
Platelets min ($x10^9$/L)	63 (19-172)	67 (13-249)	56 (3-198)	0.307
P-Fibrinogen (g/L) (n=43)	4.2 (2.0-9.6)	4.2 (2.2-6.7)	4.5 (1.5-5.7)	0.863
P-Prothrombin fragments F1+2 (pmol/l) (n=43)	542 (329-1160	583 (149-1487)	711 (284-1875)	0.811
P-D-dimer (mg/L) (n=43)	1.4 (0.6-29.6)	3.4 (0.3-13.5)	3.3 (0.8-34.0)	0.405

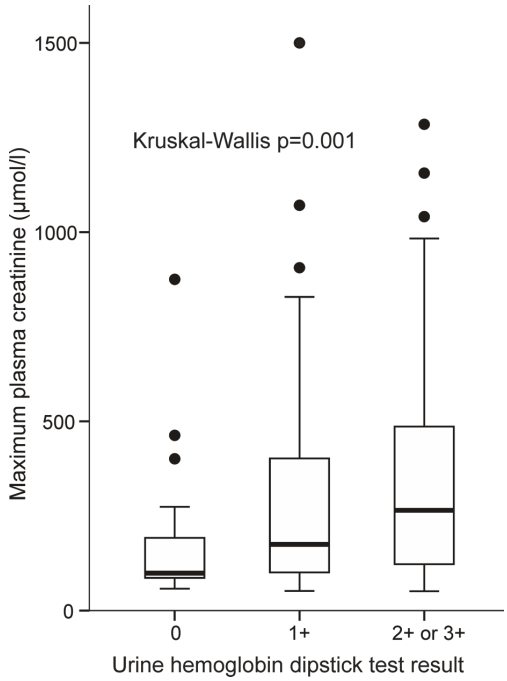
Values are medians (ranges). P=plasma, U=urine, dU=daily urine, cU=overnight urine collection, min=minimum, max=maximum.

Table II The clinical and laboratory findings in 205 patients with Puumala hantavirus infection divided into three groups according to combined positive result in urine hemoglobin and albumin dipstick tests.

	0-2+ n=58	**3-4+ n=100**	**5-6+ n=47**	**p-value**
Hospital stay (days)	5.5 (3-25)	6.0 (3-12)	7.0 (2-15)	0.022
Change in body weight (kg)	1.6 (0-10.7)	2.2 (0-10.8)	3.8 (0-12.0)	<0.001
Urinary output min (ml/day)	1900 (0-5720)	1440 (100-4900)	780 (50-7000)	<0.001
Laboratory findings in plasma and blood				
P-Creatinine max (µmol/L)	100 (52-1499)	204 (65-1071)	361 (51-1285)	<0.001
P-Urea max (mmol/L) (n=110)	9.4 (2.1-52.4	17.7 (3.9-39.7)	24.6 (2.7-52.8)	0.001
P-Sodium min (mmol/L)	135 (114-141)	132 (109-142)	129 (113-141)	<0.001
P-Alb min (g/L)	30 (11-39)	28 (22-37)	25 (18-36)	0.001
Hematocrit min	0.37 (0.22-0.43)	0.36 (0.25-0.44)	0.35 (0.25-0.46)	0.109
Leukocytes max ($x10^9$/L)	9.6 (4.2-38.6)	10.4 (5.7-31.2)	13.4 (4.4-45.0)	0.001
P-C-reactive protein max (mg/L)	78 (16-236)	81 (16-269)	80 (22-214)	0.624
Laboratory findings in urine				
dU-Protein max (g/day) (n=70)	1.2 (0.1-9.5)	1.9 (0.2-17.8)	2.0 (0.2-7.6)	0.318
cU-Albumin max (µg/min) (n=70)	97 (4-4617)	902 (12-7026)	1018 (55-4358)	0.011
cU-α1-microglobulin max (µg/min) (n=70)	20 (10-89)	25 (7-209)	27 (7-112)	0.694
U-Erythrocytes (per high power field)	2 (0-132)	4 (0-41)	14 (0-401)	<0.001
Laboratory findings reflecting hemostasis				
Platelets min ($x10^9$/L)	63 (3-249)	66 (15-198)	52 (5-187)	0.069
P-Fibrinogen (g/L) (n=43)	4.2 (2.0-9.6)	4.1 (2.8-6.7)	4.8 (1.5-5.7)	0.602
P-Prothrombin fragments F1+2 (pmol/l) (n=43)	534 (295-1160)	614 (149-1487)	769 (429-1875)	0.289
P-D-dimer (mg/L) (n=43)	1.4 (0.6-29.6)	3.6 (0.3-8.7)	3.8 (1.1-34.0)	0.113

Values are medians (ranges). P=plasma, U=urine, dU=daily urine, cU=overnight urine collection, min=minimum, max=maximum.

Figure 1. Maximum plasma creatinine level during hospital care in relation to urine hemoglobin dipstick test result at the entry to the hospital.



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Disclosure of interest

The authors report no conflicts of interest.

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PUBLICATION III

Positive urine glucose predicts the severity of acute kidney injury in Puumala hantavirus infection

Tietäväinen J, Mantula P, Outinen T, Huhtala H, Pörsti I, Niemelä O, Vaheri A, Mäkelä S, Mustonen J.

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CLINICAL RESEARCH

Glucosuria Predicts the Severity of Puumala Hantavirus Infection

Check for updates

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Introduction: Puumala hantavirus (PUUV) causes a mild type of hemorrhagic fever with renal syndrome characterized by acute kidney injury (AKI), increased capillary leakage, and thrombocytopenia. Albuminuria and hematuria in dipstick urine test at hospital admission are known to predict the severity of upcoming AKI.

Methods: We analyzed dipstick urine glucose in 195 patients with acute PUUV infection at hospital admission, and divided them into 2 categories according to the presence or absence of glucose in the dipstick urine test. Determinants of disease severity were analyzed in glucosuric and nonglucosuric patients.

Results: Altogether, 24 of 195 patients (12%) had glucosuria. The patients with glucosuria had more severe AKI than patients without glucosuria (median maximum creatinine concentration 459 μmol/l, range 78–1041 μmol/l vs. 166 μmol/l, range 51–1499 μmol/l; $P < 0.001$). The glucosuric patients had more severe thrombocytopenia (median minimum platelet count 41 $\times$ 10^9/l, range 5–102 $\times$ 10^9/l vs. 62 $\times$ 10^9/l, range 3–249 $\times$ 10^9/l; $P = 0.006$), and more pronounced signs of increased capillary leakage (change in weight, maximum plasma hematocrit, minimum plasma albumin). The glucosuric patients were more often in clinical shock at admission (20.8% vs. 1.2%; $P < 0.001$) and the length of hospital stay was longer (median 7.5 days, range 4–22 days vs. 6 days, range 2–30 days; $P = 0.009$).

Conclusion: Glucosuria is relatively rare, but when present it predicts a more severe disease course in patients with acute PUUV infection.

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KEYWORDS: acute kidney injury; glucosuria; hantavirus; Puumala virus; thrombocytopenia
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See Commentary on Page 1203

PUUV, found in Europe and Western Russia, causes a mild type of hemorrhagic fever with renal syndrome. An average of 1000 to 3000 serologically confirmed diagnoses are made annually in Finland, but up to 5% of blood donors are positive for PUUV antibodies, indicating that most of the cases are mild and remain undiagnosed.[1] The symptoms of the infection include fever, headache, nausea, abdominal pain, backache, and visual disturbances. Bleeding diathesis is rare. The renal involvement includes proteinuria, hematuria and AKI. Proteinuria is mainly albuminuria and can reach nephrotic range; however, the renal histology is that of acute tubulointerstitial nephritis.[2–4] Hospitalized patients often have AKI with transient oliguria followed by polyuria. Few patients (6%) need transient dialysis.[2] Patients show signs of increased capillary leakage and are thrombocytopenic. Case fatality is low (<0.1%)[5] and ultimate prognosis is good.

Glucosuria is a rather infrequent finding in patients with AKI. In general, glucosuria is detected in diabetic patients with high serum glucose concentration and in patients with renal tubular injury such as the Fanconi syndrome. However, 9% of adults and 12% of children who have acute PUUV infection present with glucosuria.[2,6]

We have previously shown that albuminuria and hematuria in dipstick urine test predict the severity of upcoming AKI in patients with acute PUUV infection.[7,8] We now report that glucosuria in these patients is an even stronger predictor of disease severity.

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MATERIALS AND METHODS

Subjects

The study cohort consisted of 220 adult patients treated in Tampere University Hospital, Finland, due to

serologically confirmed acute PUUV infection during the years 1994 to 2014. A detailed medical history of the patients was obtained and all patients were carefully clinically examined. Clinical features like blood pressure, heart rate, and weight were measured at least daily. The daily urine output was followed during the hospital stay.

One or several previous diagnoses had been made in 7 of 24 patients with glucosuria (29%) and in 50 of 171 patients without glucosuria (30%). The diagnoses were hypertension ($n = 13$), coronary heart disease ($n = 6$), rheumatoid arthritis ($n = 3$), bronchial asthma ($n = 6$), celiac disease ($n = 3$), prostate hyperplasia ($n = 2$), cerebral infarction ($n = 1$), juvenile rheumatoid arthritis ($n = 1$), Crohn's disease ($n = 1$), epilepsy ($n = 1$), and spherocytosis ($n = 1$). One patient had Henoch-Schönlein purpura diagnosed 25 years earlier with no kidney manifestation and 1 patient had a previously treated kidney tuberculosis. Two of the nonglucosuric patients had type 2 diabetes. One patient was pregnant and 1 was breastfeeding. None of the patients in either group had a known kidney insufficiency before the PUUV infection.

A dipstick urine test was available in 196 patients at hospital admission. One of them had a previously undiagnosed type 2 diabetes with blood glucose level of 26.9 mmol/l at admission and was excluded from further analyses. The patients were divided into 2 categories based on the presence or absence of glucose in the dipstick test. Glucosuria was detected in 24 of 195 patients (12.3%), whereas 171 of 195 patients (87.7%) were negative for glucosuria.

A chest radiograph was taken in 150 patients at hospital admission. All radiographs were analyzed by a radiologist.[9]

All patients provided a written consent and the study was approved by the Ethics Committee of the Tampere University Hospital (study codes 97166, 99256, R04180, and R09206).

Laboratory Determinations

The diagnosis of PUUV infection in 1982 to 1989 was based on duplicate samples with 4-fold or grater rise in IgG titer by the immunofluorescence assay.[10] Since 1989, recent PUUV infection was confirmed from a single serum sample by detecting the typical granular staining pattern in immunofluorescence assay[11] and/or low avidity of IgG antibodies to PUUV and /or by detecting PUUV IgM antibodies by an "in-house" enzyme-linked immunosorbent assay based on a recombinant antigen.[12] The development and use of these and other diagnostic methods have been described by Vaheri *et al*.[13]

A dipstick urine test was performed in the emergency room at hospital admission and a follow-up sample was obtained in 11/24 glucosuric and in 76/171 non-glucosuric patients during the hospital care. The dipstick urine test was obtained more than once in 4 of 24 glucosuric and 24 of 171 nonglucosuric patients. Urine dipstick analysis was made by automated tests based on refractometry using Miditron M (Roche, Basel, Switzerland) from 1997 onward, Urisys 2400 or 1900 (Roche) from 2004 onward, and Siemens (Munich, Germany) Clinitek Atlas or Advantus from 2009. The dipstick assay detects albumin, and it does not react with immunoglobulins, immunoglobulin light chains, or tubular proteins. The sensitivity of the assay to urine albumin is 0.15 to 0.3 g/l (U-Alb 1+), $\geq$ 1 g/l (U-Alb 2+) and 3 g/l (U-Alb 3+). The assay for hematuria detects heme pseudoperoxidase activity and therefore it also detects red cell casts and dysmorphic red cells. The sensitivity of the assay is approximately 10×10^6 cells/l (approximately 3–5 cells by high-power field). The dipstick test for leukocytes detects granulocyte and macrophage esterase activity. The sensitivity is approximately 30×10^6 cells/l (1–2 cells by high-power field). The dipstick test for glucose detects glucosuria from glucose level 3 to 5 mmol/l upward. Glucosuria 3+ corresponds to a glucose level exceeding 30 mmol/l.

Plasma creatinine was analyzed by Vitros (Johnson & Johnson, Rochester, NY) until the 1999 and by Cobas Integra (F. Hoffmann-La Roche Ltd., Basel, Switzerland) from thereafter. Plasma creatinine value was determined daily during the hospital stay and the first value and the highest value (maximum) were taken into the statistical analysis. In addition, several other blood or plasma samples were analyzed on a daily basis including blood cell count, C-reactive protein, and electrolytes. Plasma creatinine max and blood cell count were available from all patients. Creatinine first was available from 24 of 24 glucosuric and 167 of 171 nonglucosuric patients. C-reactive protein was available from 22 of 24 glucosuric and 169 of 171 nonglucosuric patients. Maximum plasma urea concentration was available from 20 of 24 of the glucosuric and 85 of 171 nonglucosuric patients and minimum plasma albumin from 19 of 24 glucosuric and 86 of 171 nonglucosuric patients. Daily urinary volume was available from all glucosuric and from 163 of 171 nonglucosuric patients. Blood cell count was determined by automated hematological cell counters (Bayer Diagnostics, Elkhart, IN), and sodium, potassium, urea, and albumin concentrations using routine automated chemistry analyzers. All laboratory determinations were performed by the Laboratory Centre of the Pirkanmaa Hospital District (later named Fimlab laboratories), Tampere, Finland. Minimum or maximum of

Table 1. Clinical findings in 195 patients with PUUV infection according to the presence of glucosuria at admission

	Glucosuria $n = 24$		No glucosuria $n = 171$		
	Median/number	Range/%	Median/number	Range/%	*P* value
Age	40	25–67	41	21–73	0.908
Men	21	87.5	111	64.5	0.034
BMI	28.1	20.4–37.2	25.6	18.5–41.9	0.031
Shock at admission[a]	5	20.8	2	1.2	<0.001
First systolic BP (mm Hg)	115	70–167	124	72–210	0.025
Max systolic BP (mm Hg)	144	123–204	138	95–210	0.016
Max diastolic BP (mm Hg)	94	78–111	87	60–120	0.017
Min systolic BP (mm Hg)	109	60–150	113	68–107	0.243
Min diastolic BP (mm Hg)	75	36–91	70	39–100	0.542
Hospital stay (d)	7.5	4–22	6	2–30	0.009
Weight change (kg)[b]	4.0	0.5–11.3	2.5	0.1–12.0	0.025
Urinary output min (ml/d)	600	0–4900	1450	0–7000	0.001
Dialysis	3	12.5	7	4.1	0.110

BMI, body mass index; BP, blood pressure; Max, maximum; Min, minimum; PUUV, Puumala hantavirus.
[a]Shock was defined as systolic blood pressure less than 90 mmHg and symptoms of shock.
[b]Difference between highest and lowest weight during hospital care reflecting both capillary leakage and fluid accumulation during oliguric phase.
Glucosuria: urine dipstick glucose from 1+ to 3+; No glucosuria: urine dipstick glucose 0.

the values were taken to the statistical analysis as indicated in Tables 1 and 2.

Statistical Analysis

For the descriptive analyses, the medians with ranges and frequencies (*n*) with percentages and cross-tabulation were used for the exploratory analyses. For the comparative analyses, Mann-Whitney *U* test and Student's *t*-test for independent samples were performed. The χ^2 tests were used to examine differences in proportions. The Spearman correlations (r_S) were used to study the relationship between variables. Binary logistic regression was used to adjust upcoming severe AKI with plasma creatinine at admission. All analyses were performed using IBM SPSS Statistics version 24 (IBM, Armonk, NY).

RESULTS

Clinical, Laboratory, and Radiological Findings

Glucosuria was present in 24 of 195 patients (12.3%) with acute PUUV infection. In most cases, glucosuria was mild. Twenty patients presented with urine glucose 1+ in the dipstick test, 3 patients with urine glucose 2+, whereas only 1 patient had urine glucose 3+. Table 1 shows the clinical data of the patients with and without glucosuria. We found glucosuria in 21 of 132 (15.9%) men but in only 3 of 63 (4.8%)

Table 2. Laboratory findings in 195 patients with PUUV infection according to the presence of glucosuria at admission

	Glucosuria $n = 24$		No glucosuria $n = 171$		
	Median	Range	Median	Range	*P* value
Plasma and blood findings					
Creatinine first (μmol/l)	184	72–617	96	43–1113	0.002
Creatinine max (μmol/l)	459	78–1041	166	51–1499	<0.001
Urea max (mmol/l)	30.3	3.2–39.3	17.4	2.1–54.5	0.012
Platelets min ($\times 10^9$/l)	41	5–102	62	3–249	0.006
Hematocrit max	0.51	0.34–0.60	0.43	0.33–0.59	<0.001
Albumin min (g/l)	24	11–33	28	20–39	<0.001
Leukocytes max ($\times 10^9$/l)	16.0	5.7–45	10.2	4.2–35.5	<0.001
CRP max (mg/l)	81	21–213	77	16–269	0.446
Sodium min (mmol/l)	128	122–140	132	109–142	0.001
Sodium max (mmol/l)	143	132–159	141	128–150	0.034
Potassium min (mmol/l)	3.6	3.7–5.2	3.6	3.3–5.5	0.554
Potassium max (mmol/l)	4.4	2.7–4.0	4.3	2.9–4.9	0.306
Glucose (mmol/l)[a]	8.3	4.9–17.6	5.9	3.6–10.9	0.001
Urine findings					
Leukocyte count ($\times 10^6$/l)	4	0–195	3	0–86	0.442
Erythrocyte count ($\times 10^6$/l)	11	2–254	4	0–401	<0.001

CRP, C-reactive protein; Max, maximum; min, minimum; PUUV, Puumala hantavirus.
[a]Blood glucose concentration at hospital admission.
Glucosuria: urine dipstick glucose from 1+ to 3+; No glucosuria: urine dipstick glucose 0.

women ($P = 0.034$). None of the glucosuric patients had a diagnosis of diabetes. Patients who were nonglucosuric at hospital admission were nonglucosuric in the follow-up samples. One glucosuric patient was positive for urine glucose at day 1 after hospital admission but turned urine glucose negative in the following samples. The rest of the glucosuric patients were negative for urine glucose in the follow-up samples. The patients arrived at the hospital a median of 4 days after the onset of fever with no difference between the groups.

The use of drugs potentially influencing blood glucose concentration was studied and we found no differences in the use of beta blockers, thiazide diuretics, or corticosteroids (oral or inhaled) or in the use of antibiotics between the groups (data not shown). The glucosuric patients were more often in clinical shock at admission, but presented with higher maximum systolic and diastolic blood pressure during the hospital stay, the length of which was also longer than in nonglucosuric patients, which can be assumed to reflect the overall severity of the disease (Table 1). There was no difference in smoking habits between the groups (data not shown).

The laboratory findings of the patients according to the presence of glucosuria are presented in Table 2. The first plasma creatinine measured at admission was higher in glucosuric patients. Patients with glucosuria had higher maximum plasma creatinine and urea concentrations. When adjusted to creatinine at admission, glucosuria remained a significant predictor of severe AKI, defined as plasma creatinine $\geq$ 353.6 μmol/l; odds ratio 5.9 (95% confidence interval 1.9–18.0).[14] The association of glucosuria with higher maximum plasma creatinine level was observed in both sexes (data not shown). The minimum number of platelets was also lower in glucosuric patients (Table 2).

The presence of glucosuria was related to variables reflecting capillary leakage. The patients with glucosuria had a greater change in weight (Table 1), higher maximum hematocrit, and lower minimum albumin (Table 2) when compared with patients without glucosuria.

Blood glucose sample taken at the hospital admission was available in 14 of 24 patients with glucosuria and in 62 of 171 patients without glucosuria. The concentration of blood glucose was higher in the glucosuric patients (Table 2). Importantly, blood glucose level was below the kidney glucose threshold of 10 mmol/l in 60/62 nonglucosuric and in 12 of 14 glucosuric patients. Blood glucose sample taken later during the hospital care was available in 15 glucosuric and in 84 nonglucosuric patients. The level of blood glucose decreased and there was no difference in the minimum blood glucose concentration between the groups (data not shown).

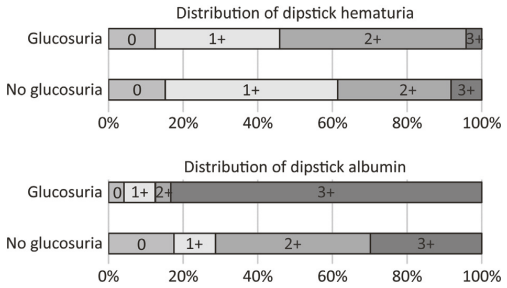

Figure 1. Distribution of findings in urine dipstick test for hematuria (U-Eryt) and albuminuria (U-Alb) in patients with acute Puumala hantavirus infection as classified to subgroups according to the presence or absence of glucosuria. Glucosuric patients, $n = 24$; nonglucosuric patients, $n = 171$.

There was no correlation between body mass index (BMI) and blood glucose concentration at hospital admission either in patients with glucosuria or in patients without glucosuria ($r_S = 0.396$, $P = 0.180$ and $r_S = 0.178$, $P = 0.203$, respectively). BMI did not correlate with maximum plasma creatinine value either in glucosuric ($r_S = 0.062$, $P = 0.784$) or in nonglucosuric patients ($r_S = 0.040$, $P = 0.623$).

The number of urine erythrocytes was higher in glucosuric patients (Table 2); however, there was no difference in dipstick hematuria between the groups (Figure 1). The prevalence and the amount of albumin in the dipstick urine test at hospital admission was higher in glucosuric patients. Dipstick albumin was positive in 96% of patients and reached 3+ in 83% of patients with glucosuria compared with 83% and 30% of patients without glucosuria, respectively ($P < 0.001$ for both) (Figure 1).

The chest radiography was abnormal in 6 of 14 (43%) patients with glucosuria and in 25 of 136 (18%) patients without glucosuria ($P = 0.042$). There was no difference in the occurrence of pleural effusion (data not shown).

Dipstick Test and the Incidence of AKI

We have previously reported that albuminuria and hematuria in dipstick urine test predict the severity of the upcoming AKI.[8] Here we evaluated the impact of dipstick glucosuria, albuminuria, hematuria, and combined albuminuria + hematuria + glucosuria, in predicting the severity of the upcoming AKI.

When evaluating the impact of glucosuria, the median maximum creatinine level was higher in patients with glucosuria than in patients without glucosuria (459 μmol/l, range 78–1041 vs. 166 μmol/l, range 51–1499, $P < 0.001$) (Figure 2a).

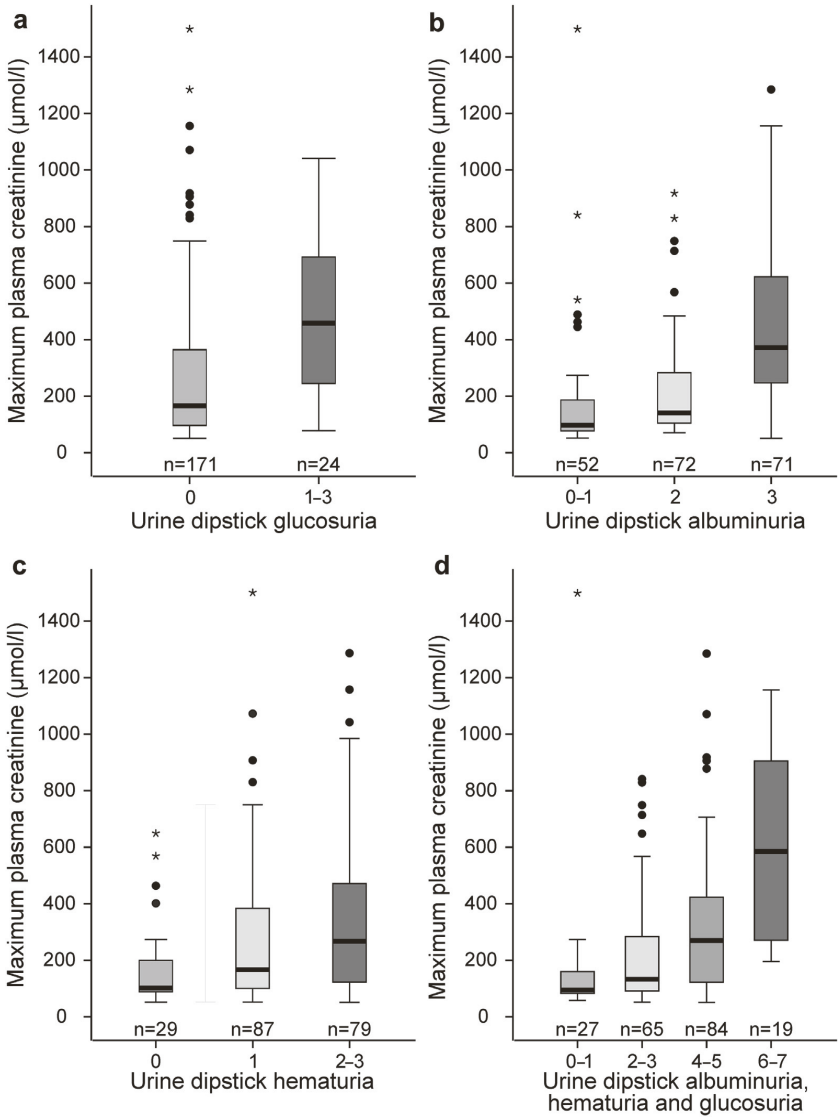

Figure 2. Maximum plasma creatinine value analyzed according to urine dipstick result: glucosuria (a), albuminuria (b), hematuria (c), and combined result of albuminuria + hematuria + glucosuria (d). The number of patients in each group is indicated in the figure. Boxplots are with median, interquartile range, minimum, and maximum within 1.5 interquartile range, and outliers are displayed as circles and extreme values as asterisks.

When assessing the significance of albuminuria, increasing positive result in dipstick urine albumin test predicted higher maximum creatinine level. The median maximum creatinine level was 98 µmol/l (range 52–1499) for albumin 0–1+, 142 µmol/l (range 71–918) for albumin 2+, and 372 µmol/l (range 51–1285) for albumin 3+ (Figure 2b).

When evaluating the impact of hematuria, increasing positive result in the dipstick test also predicted higher maximum creatinine level. The median for maximum plasma creatinine was 102 µmol/l (range 58–648) for 0+; 166 µmol/l (range 52–1499) for 1+; and 267 µmol/l (range 51–1285) for 2 to 3+ (Figure 2c).

Finally, higher positive result in the dipstick test for albuminuria + hematuria + glucosuria predicted higher maximum creatinine. The median maximum creatinine was 95 µmol/l (range 58–1499) for 0 to 1+,

133 μmol/l (range 52–841) for 2 to 3+, 271 μmol/l (range 51–1285) for 4 to 5+, and 585 μmol/l (range 196–1156) for 6 to 7+ (Figure 2d). There were no patients with a sum of positive results exceeding 7+.

DISCUSSION

To our knowledge, this is the first study to show that glucosuria predicts the severity of the upcoming AKI and the overall severity of disease in patients with acute hantavirus infection. Glucosuria correlated with higher systolic and diastolic blood pressure during the hospital stay, which most likely reflects the severity of AKI and fluid retention during the oliguric phase of the infection. Glucosuria correlated with the determinants of capillary leakage: low blood pressure at hospital admission, presence of clinical shock and pathologic chest radiography changes, high hematocrit, low plasma albumin, and greater change in weight during the hospital stay. Glucosuria also correlated with thrombocytopenia. The length of the hospital stay was longer in the glucosuric patients, reflecting the overall severity of the disease. Therefore, glucosuria at admission correlated with all of the main clinical findings of severe PUUV infection: AKI, inflammation, capillary leakage, and thrombocytopenia.

In the current study, 12% of the patients presented with glucosuria at hospital admission. Glucosuria was more common in men than in women, but the same relation between glucosuria and more severe AKI was seen in both sexes. Nonglucosuric patients remained glucose negative in all follow-up samples. One glucosuric patient was glucosuria positive in the first follow-up sample but turned glucosuria negative later during the hospital care. All the rest of the glucosuric patients were glucosuria negative in the follow-up urine samples. It appears that glucosuria is an early and transient sign in PUUV infection.

Although plasma creatinine was higher in glucosuric patients already at the time of the first creatinine measurement, it increased more than in nonglucosuric patients during the hospital care. When the outcome was severe AKI and the result was adjusted to the first plasma creatinine measured, glucosuria still remained a significant predictor of severe AKI. Therefore, glucosuria both associates with and predicts severe AKI. The patients arrived at hospital a median of 4 days after the onset of fever and there was no difference between the groups.

In general, glucosuria most often results from high blood glucose concentration. Glucose is freely filtered into primary urine but it is completely reabsorbed in the tubules. Ninety percent of the reuptake takes place in the proximal tubule by the sodium-glucose cotransporter type 2/glucose transporter 2 (SGLT2/GLUT2) (low affinity–high capacity system). The convoluted part of the proximal tubule also participates in glucose reuptake via the SGLT1/GLUT1 (high affinity-low capacity system). The kidney tubular glucose threshold for blood glucose is approximately 10 to 11 mmol/l at normal glomerular filtration rate, after which the reabsorptive capacity is overwhelmed and glucose appears in the urine.[15] When glomerular filtration rate increases, like in the case of pregnancy, the glucose threshold level decreases. Conversely, in chronic renal insufficiency, the level of glucose threshold increases and higher blood glucose levels are tolerated before glucose appears in the final urine. In addition, in diabetes, the maximum reabsorptive capacity of the kidney increases and therefore hyperglycemic diabetic patients may not be glucosuric.[15] Little is known about glucosuria in AKI caused by etiologies other than acute tubulointerstitial nephritis (TIN).

We found that blood glucose concentration at hospital admission was higher in the glucosuric than in the nonglucosuric patients. However, none of the glucosuric patients had diabetes. Also, there was no difference in the use of drugs that can influence blood glucose concentration. During the hospital stay, the blood glucose levels decreased and there was no difference in the minimum blood glucose concentration between the groups. Further, at admission, the median blood glucose level in the glucosuric patients was 8.3 mmol /l, which is below the threshold level of a healthy kidney and should not result in glucosuria. It appears that glucosuria detected in the present study was caused by kidney damage and not by hyperglycemia. This is in line with the results obtained in animal studies in which glycerol-induced acute renal failure and proximal tubular damage resulted in glucosuria in normoglycemic rats.[16]

In the present study, BMI was higher in glucosuric patients but BMI did not correlate with either blood glucose level at hospital admission or maximum plasma creatinine value. Therefore, BMI does not explain the glucosuria or the observed association between glucosuria and severe AKI. It is possible that the higher blood glucose concentration in the glucosuric patients was due to the hypercortisolism caused by the acute PUUV infection,[17] and that cortisol raised blood glucose more in the obese patients. However, cortisol metabolism was not systematically analyzed in this study.

Glucosuria can result from proximal tubular damage, such as the Fanconi syndrome. PUUV is known to infect tubular epithelial cells and histologically PUUV infection causes an acute TIN.[4] In adult patients, 60% to 80% of TIN cases are drug induced, the second most

common cause being autoimmune diseases.[18] However, glucosuria is not a common finding in acute TIN in adults. Some causative agents are more likely than others to induce glucosuria in acute TIN. Among these are some non–β-lactam antibiotics, antiviral agents, cytostats, the antiepileptic sodium valproate, and the mood stabilizer lithium.[19,20] In addition, D-serine, a newly acknowledged uremic toxin, can cause damage to the proximal tubule and glucosuria.[21] As a comparison, 100% of pediatric patients with TIN and uveitis syndrome had glucosuria.[22] The etiology of TIN and uveitis syndrome, remains unknown.

Several infections can cause TIN. In pediatric patients with Yersinia pseudotuberculosis–induced interstitial nephritis, 27% of patients with AKI exhibited glucosuria, whereas none of the patients with normal kidney function had glucosuria.[23] In a small case series from the Czech Republic, all 3 pediatric patients with PUUV infection had glucosuria and AKI.[24] In our previous reports from patients with acute PUUV infection, 9% of adults and 12% of children had glucosuria.[2,6] However, to our knowledge, this is the first time glucosuria has been used as a maker of disease severity in infection-induced AKI.

The PUUV typically infects kidney tubular epithelial cells. The PUUV RNA is mostly seen in the distal tubuli[25] and several adhesion molecules and cytokines are seen in the peritubular area of the distal nephron.[26] Subsequent splitting of the tubular basement membrane has been detected in both proximal and distal tubuli.[27] The proximal tubule is essential in the reuptake of glucose. In addition, inflammation and cytokine release influence kidney function by decreasing renal tissue perfusion and glomerular filtration rate, decreasing the expression of SGLT2, SGLT3, and GLUT 2, and increasing fractional glucose excretion.[28] Several different cytokines are also known to influence the severity of PUUV infection.[29–31] As in a case of glucosuria in experimental animal study,[16] glucosuria in our study is most likely a marker of a more severe tubular injury in patients with PUUV infection. Whether glucosuria is a marker of unspecific damage to the proximal tubular cells or a result of a specific pathology needs further investigation.

The prevalence and the amount of albuminuria were higher in patients with glucosuria than in patients without glucosuria (Figure 1). Although urine dipstick hematuria did not differ between the study groups, the number of urine erythrocytes was higher in the glucosuric patients (Table 2). The reason for this discrepancy is unknown.

We previously reported that albuminuria, hematuria, and combined albuminuria + hematuria predicted the severity of the upcoming AKI.[7,8] We now evaluated how different variables or combination of variables in the dipstick urine test predict AKI. Glucosuria is rare, but the presence of even mild glucosuria associates and predicts AKI (Figure 2a). Albuminuria and hematuria are more common and they have a dose-dependent effect on the severity of AKI. Mild albuminuria and mild hematuria indicate benign disease course, whereas abundant albuminuria and hematuria predict severity of AKI (Figure 2b and c); however, the maximum creatinine even with abundant albuminuria or hematuria is still lower than in glucosuric patients. In the combination of albuminuria, hematuria, and glucosuria, there appears to be a threshold level of 4+ (Figure 2d), which separates patients at greater risk of developing severe AKI.

Glucosuria, as well as albuminuria and hematuria, predict the overall severity of the infection. It is of interest, however, that only glucosuria associates with the level of thrombocytopenia.

In conclusion, we show here for the first time that glucosuria in patients with acute hantavirus infection is a good marker for the severity of upcoming AKI and the overall course of the PUUV infection.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)
Strobe Statement.

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PUBLICATION IV

Flash-like albuminuria in acute kidney injury caused by Puumala hantavirus infection

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Article

Flash-Like Albuminuria in Acute Kidney Injury Caused by Puumala Hantavirus Infection

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Abstract: Transient proteinuria and acute kidney injury (AKI) are characteristics of Puumala virus (PUUV) infection. Albuminuria peaks around the fifth day and associates with AKI severity. To evaluate albuminuria disappearance rate, we quantified albumin excretion at different time points after the fever onset. The study included 141 consecutive patients hospitalized due to acute PUUV infection in Tampere University Hospital, Finland. Timed overnight albumin excretion (cU-Alb) was measured during the acute phase in 133 patients, once or twice during the convalescent phase within three months in 94 patients, and at six months in 36 patients. During hospitalization, 30% of the patients had moderately increased albuminuria (cU-Alb 20–200 μg/min), while 57% presented with severely increased albuminuria (cU-Alb >200 μg/min). Median cU-Alb was 311 μg/min (range 2.2–6460) ≤7 days after fever onset, 235 μg/min (range 6.8–5479) at 8–13 days and 2.8 μg/min (range 0.5–18.2) at 14–20 days. After that, only one of the measurements showed albuminuria (35.4 μg/min at day 44). At six months, the median cU-Alb was 2.0 μg/min (range 0.6–14.5). Albuminuria makes a flash-like appearance in PUUV infection and returns rapidly to normal levels within 2–3 weeks after fever onset. In the case of AKI, this is a unique phenomenon.

Keywords: albuminuria; proteinuria; Puumala virus; hantavirus; acute kidney injury; hemorrhagic fever with renal syndrome; HFRS

1. Introduction

The most common hantavirus infection in Europe is hemorrhagic fever with renal syndrome (HFRS) caused by Puumala virus (PUUV) [1]. The reservoir host of this hantavirus is the bank vole (*Myodes glareolus*), the excreta of which contain the infectious viruses. The virus is transmitted to humans via the inhalation of aerosols contaminated with the virus. Many other hantaviruses, including Dobrava–Belgrade, Sochi and Seoul viruses, are also found in Europe. They cause diseases of varying severity, whereas PUUV predominantly causes a mild form of HFRS. The most severe form of hantavirus infection, the hantavirus cardiopulmonary syndrome (HCPS), occurs in the Americas [2].

After an incubation period of at least two weeks, the symptoms of PUUV infection. Including fever, headache, and back and abdominal pain, occur [3]. The main affected organ is the kidney [4,5].

Soon after the beginning of fever, patients typically present with proteinuria, which reaches the nephrotic range in ~30% of patients [4,6]. Other signs of acute nephritis, including hematuria (in 60–90% of the patients) and rising plasma creatinine, are seen early in the course of the disease, concurrently with thrombocytopenia [4]. Along with predominant acute kidney injury (AKI), clinical signs of capillary leakage, i.e., an acute rise in hematocrit, pleural effusion, ascites, and perirenal fluid accumulation, are often seen [3]. The diagnosis of the disease is based on typical clinical signs and confirmation by serological testing [7].

The care of the disease is supportive, as no specific treatment for PUUV infection exists. The prognosis of the kidney involvement is favorable and recovery from AKI starts when typical polyuria evolves. As a rule, kidney function recovers to normal even after severe AKI, which is seen in ~30% of cases [8]. The need for hemodialysis treatment has been reported to be around 4% [8]. The rare fatal cases (0.1–0.4%) show serious signs of capillary leakage with pulmonary edema and circulatory shock [8]. Severe bleeding complications related to thrombocytopenia are rare [9,10].

While the clinical course of PUUV infection has been well characterized, the pathogenesis of kidney involvement has remained poorly known. Recently, an association was reported between the higher amount of proteinuria and more severe AKI, which raises the question of a possible link between the mechanisms of proteinuria, capillary leakage and AKI in this disease [5,11]. Many mediators of inflammation react in acute PUUV infection. In our earlier studies, the higher urinary excretion of interleukin (IL)-6 and soluble form of urokinase-type plasminogen activator receptor (suPAR), as well as a higher plasma adipocytokine resistin concentration, correlated with the amount of albuminuria [12–14].

In our previous study of 70 patients with acute PUUV infection, albuminuria peaked around the fifth day after the beginning of the fever, i.e., 4–5 days before the maximum values of plasma creatinine [6]. There was a clear decline in urine albumin excretion during the hospital stay, but the disappearance of albuminuria was not systemically studied in these patients. Eleven days after the beginning of the fever, three out of six patients still presented with significant albuminuria [6]. Corresponding results were observed in a cohort of 36 patients with acute PUUV infection, in whom a sharp decrease in urine albumin excretion was detected during hospital care [15]. However, at the end of the follow-up, some patients still had albuminuria twelve days after the beginning of the fever [15]. Although earlier reports have shown that long-standing albuminuria is not a typical finding after PUUV-induced AKI [8,16], the timing of the disappearance of albuminuria after acute PUUV infection has not been systemically examined.

The aim of this study was to investigate the disappearance rate of urinary albumin excretion in the course of acute PUUV infection. We examined whether some patients would present with long-lasting albuminuria in order to elucidate the possible underlying factors. We found that regardless of the amount of albuminuria or AKI severity during the acute phase of PUUV infection, albuminuria disappears within 2–3 weeks. Such rapid changes probably reflect the quick alterations in the permeability of the glomerular filtration barrier, an exceptional phenomenon when considering the present knowledge from the other types of AKI.

2. Results

The clinical characteristics and laboratory findings of the 141 PUUV-infected patients in the acute phase are shown in Table 1. Out of 141 patients, 44 (31%) had severe AKI, and seven (5%) needed dialysis treatment. Four patients (3%) had symptoms of shock and systolic blood pressure lower than 90 mmHg at admission. One patient had a concurrent acute Guillain–Barré syndrome, probably caused by the PUUV infection, and was treated with plasma exchanges.

Table 1. Clinical and laboratory findings during hospitalization in 141 patients with acute Puumala hantavirus infection.

Finding	Median	Range
Length of hospital stay (days)	6	2–22
Change in body weight during hospital stay (kg)	2.0	0–12.0
Systolic blood pressure on admission (mmHg)	124	70–210
Plasma creatinine max [1] (μmol/L)	185	51–1499
Hematocrit max [1]	0.44	0.33–0.60
Platelets min [2] ($\times 10^9$/L)	61	8–238
Plasma sodium min [2] (mmol/L)	130	109–141
Plasma potassium max [1] (mmol/L)	4.2	3.3–5.5
Blood leukocytes max [1] ($\times 10^9$/L)	10.5	3.9–45.0
Plasma C-reactive protein max [1] (mg/L)	79	16–269
Plasma albumin min [2] (g/L) n = 45	26	11–36

[1] max, maximum value during hospitalization; [2] min, minimum value during hospitalization.

Among the 141 consecutive patients, the acute phase urine sample was missing in eight patients due to anuria or technical reasons. Their urine samples were analyzed in the later course of the disease. The acute phase cU-Alb in 133 (94%) patients was measured on the median seventh (range 3–17) day after the beginning of symptoms, i.e., fever. In the acute phase, 40 (30%) patients had cU-Alb 20–200 μg/min. Severely increased albuminuria (cU-Alb 200–1200 μg/min) was detected in 50 (38%) patients and nephrotic range albuminuria (cU-Alb >1200 μg/min) in 26 (20%) patients. Among those who had cU-Alb >200 μg/min, the sample was taken at a median of 7 (range 3–13) days after the onset of fever.

During the hospital stay in the acute phase, 38 patients had only a mild or no clear rise in plasma creatinine concentration, defined by the maximum plasma creatinine under 100 μmol/L. In this group, median cU-Alb was 66.4 (range 2.2–1460) μg/min. Of them, 10 patients had severely increased albuminuria (cU-Alb >200 μg/min) including one subject who had nephrotic range albuminuria (cU-Alb 1460 μg/min).

After hospital discharge, cU-Alb was measured once or twice in 94 patients in the convalescent phase and in 36 patients at six months. The amount of albuminuria at different time points after the beginning of fever is shown in Table 2. At 14–20 days after the beginning of fever, none of the patients had significant albuminuria anymore. The disappearance of albuminuria is shown in the Figure 1.

Table 2. The amount of overnight albuminuria measured at the different time points after the onset of symptoms, i.e., beginning of the fever, in 141 Puumala virus (PUUV)-infected patients.

Days after the Beginning of Fever	≤7 Median 6 Days	8–13 Median 9 Days	14–20 Median 19 Days	21–30 Median 24 Days	31–40 Median 38 Days	41–60 Median 46 Days	6 Months
	n = 77	**n = 56**	**n = 41**	**n = 32**	**n = 27**	**n = 47**	**n = 36**
cU-Alb, μg/min median (range)	311.4 (2.2–6460.7)	234.9 (6.8–5479.2)	2.8 (0.5–18.2)	2.5 (0.4–18.4)	2.1 (0.3–10.1)	2.9 (0.2–35.4)	2.0 (0.6–14.5)

The amount of albuminuria during hospitalization did not have an effect on the disappearance rate, as shown in Table 3, where the timing and the number of patients at each time point are presented according to the amount of albuminuria in the acute phase. The effect of AKI severity was evaluated in 44 patients with severe AKI, in whom 19 patients' cU-Alb was controlled within 28 days after the beginning of fever. Those 19 patients had median cU-Alb 337.9 (37.3–6460.7) μg/min at the acute phase, but none of them had significant albuminuria at the first control visit (median cU-Alb 5.5 μg/min, ranging from 1.0 to 18.2 μg/min). Later in the convalescent phase, only one patient had moderately increased albuminuria (cU-Alb 35.4 μg/min, at day 44 after the onset of fever). This patient did not take part in the other follow-up visits and the urine sample was missing in the acute phase due to technical reasons. The maximum plasma creatinine during hospitalization in this patient was 138 μmol/L.

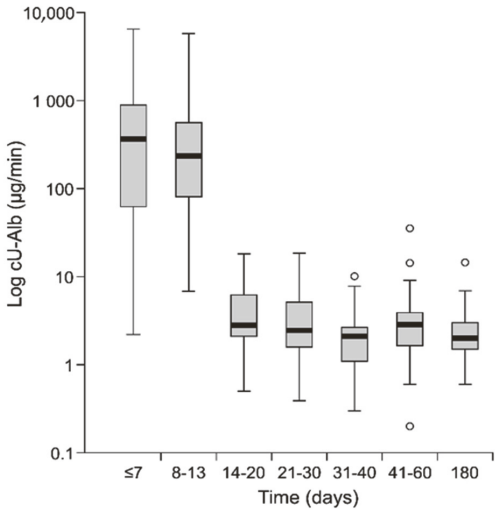

Figure 1. The decrease in overnight albuminuria according to the days after the onset of the fever in 141 patients. Boxplots are with the median (thick line), interquartile range (box), minimum, and the maximum within a 1.5 interquartile range (whiskers). Outliers are displayed as circles. The values of the timely collected overnight excretion of urinary albumin (cU-Alb) are log transformed.

Table 3. The amount of albuminuria at the control visits in 133 PUUV-infected patients categorized by the amount of albuminuria during hospitalization according to the time after the onset of symptoms, i.e., beginning of the fever.

		Days after the Beginning of Fever				
cU-Alb Categories	**cU-Alb during Hospitalization, Median (Range)**	**14–20 Median 19 Days**	**21–30 Median 24 Days**	**31–40 Median 38 Days**	**41–60 Median 45 Days**	**6 Months**
		Number of Patients and cU-Alb at Control Visits, Median (Range)				
	n = 133	**n = 41**	**n = 31**	**n = 24**	**n = 44**	**n = 34**
<20 µg/min	n = 17 11.4 (2.2–19.0)	n = 7 3.5 (2.4–9.0)	n = 4 2.1 (1.9–2.5)	n = 4 2.0 (1.0–4.6)	n = 3 1.5 (0.8–1.8)	n = 6 1.8 (1.4–6.9)
>20–200 µg/min	n = 40 86.4 (21.4–198.4)	n = 19 2.2 (0.5–14.6)	n = 5 1.8 (0.4–8.0)	n = 10 2.4 (0.7–6.3)	n = 15 3.2 (0.2–5.4)	n = 9 1.5 (1.1–5.4)
>200–1200 µg/min	n = 50 410.1 (203.1–974.8)	n = 8 4.6 (0.8–14.5)	n = 14 2.4 (0.5–18.4)	n = 7 1.8 (0.5–7.8)	n = 19 2.6 (1.0–7.8)	n = 11 2.6 (0.6–14.5)
>1200 µg/min	n = 26 1981.1 (1202.5–6460.7)	n = 7 3.8 (1.3–18.2)	n = 8 4.9 (1.0–8.0)	n = 3 2.5 (2.1–10.1)	n = 7 3.3 (1.7–14.3)	n = 8 2.0 (1.3–3.1)

In the acute phase, only three (2%) patients had an U-Alb value lower than the detection limit (3 mg/L). After hospitalization and at six months, 109/147 (74%) and 23/36 (64%) of the U-Alb measurements were under the detection limit, respectively.

3. Discussion

The present data show that albuminuria, detected in 89% of the patients in this cohort, disappears rapidly, within two to three weeks after acute PUUV infection. This was observed even in patients with severely increased albuminuria, as well as in patients with severe AKI. To our knowledge, the rate of decline of albuminuria shortly after PUUV infection has not been systematically studied before.

In the acute PUUV infection, the rapid onset of urinary albumin excretion to several grams per day reflects the changes in the barrier function of the glomeruli. A previous analysis of urinary proteins in this disease indicated that the protein is mostly albumin but also larger proteins like IgG are found in the urine, suggesting proteinuria of glomerular origin [17]. The elevated levels of lower

molecular weight proteins, α_1-microglobulin and β_2-microglobulin, are also found in the urine of PUUV patients reflecting impaired tubular reabsorption capacity [17,18]. In a recent study, also high amounts of free immunoglobulin light chains were detected in the urine during hospitalization in acute PUUV infection [19]. Of note, temporary proteinuria of such a magnitude is not typical for acute tubulointerstitial nephritis (ATIN), which is the most common histological finding in PUUV infection [4].

Endothelial dysfunction and the increased permeability of the capillary wall are characteristic findings during PUUV infection that may affect several organs. One third of the patients have pulmonary infiltrates in chest radiography [20]. Pleural and pericardial fluid and the swelling of the kidneys are signs often found in ultrasound examinations [5,21,22]. Although hantavirus enters the endothelial cells via β_3 integrin, and endothelial cells in the capillaries of various organs are the main site of hantavirus replication, the virus does not seem to cause direct cytopathic effects [23,24]. This finding corresponds to the strikingly favorable prognosis of kidney function in the course of PUUV infection. Renal biopsy samples in PUUV infection show only minimal glomerular changes, and the main findings include interstitial inflammation, edema and cell-infiltrates, with accompanying acute tubular necrosis to a varying extent (14–88%) [25–27]. In a large biopsy material, the histological findings correlated only slightly with the severity of AKI [27]. The precise mechanisms of AKI during acute PUUV infection still remain unresolved.

In other kidney diseases with transient nephrotic range proteinuria, like minimal change disease, a podocytopathy of the glomeruli is often present [28]. A disturbance in the integrity of the glomerular capillary wall during PUUV infection is supported by the results of electron microscopy studies of kidney biopsies taken in the acute phase of the disease [26,29]. Recently, Boehlke et al. reported a patient with acute PUUV infection, biopsied around the seventh day after the onset of fever. The renal biopsy sample showed the effacement of the podocyte foot processes and the loss of slit diaphragms [30]. Furthermore, changes in proteins related to cell-to-cell tight junctions have been reported in the course of acute hantavirus infection [31]. PUUV has been shown to infect podocytes, induce modulations in their cytoskeleton, and impair the migration and adhesion capacity of renal cells in vitro [24,32].

The virulence factors of hantavirus infections and the mediators that are responsible for the varying disease severity remain unknown. Nevertheless, the inflammatory responses of the host are considered important. A multitude of inflammatory mediators are probably activated during the infection, while certain human leukocyte antigen (HLA) -types are associated with a more severe disease during PUUV infection [33]. However, proteinuria of a corresponding magnitude is not typical for many other inflammatory diseases like septic kidney injury. Whether some inflammation factors have a pathogenic role in the emerging proteinuria during PUUV infection remains unclear. Previously, urinary-IL-6 level correlated with the amount of albuminuria in PUUV-infected patients in the acute phase, while in parallel, the plasma level of IL-6 was moderately elevated [12]. The level of urinary suPAR in PUUV-infected patients correlated with the amount of albuminuria, and simultaneously, the plasma concentration of suPAR was also markedly increased [13]. As neither the amount of IL-6 nor suPAR in the urine correlated with their plasma concentrations, these mediators were probably also produced locally in the kidneys. Moreover, high plasma levels of the adipocytokine resistin correlated with the amount of albuminuria, determined by urine dipstick analysis at hospital admission, and also with the severity of the upcoming AKI in PUUV-infected patients [14]. Resistin is known as a marker of macrophage activation and considered as a possible link between AKI and inflammatory responses [34]. The site of excess resistin synthesis during PUUV infection remains unknown. Whether a specific inflammatory mediator could cause albuminuria in PUUV infection is not clear. Of note, nephrotic range proteinuria is a rare finding during other infections and inflammatory diseases accompanied with AKI.

In PUUV infection, the flash-like appearance of albuminuria and the association between albuminuria quantity with upcoming AKI severity [6,11] are unique findings in the context of AKI. In addition, the amount of hematuria associates with the severity of AKI in PUUV infection [35].

The presence of glucosuria, detected in 12% of the patients, was a strong predictive factor not only for clinical shock, but also the severity of AKI in acute PUUV infection [36]. As the current definition of AKI by the committee of Kidney Disease Improving Global Outcomes (KDIGO) leaves urine analysis unacknowledged, it is difficult to compare the PUUV-induced AKI with other forms of AKI [37]. Altogether, AKI comprises a heterogenic group of conditions and the pathogenesis may not be totally consistent. Kidney diseases accompanied with nephrotic range proteinuria are usually related with normal or chronically reduced glomerular filtration rate and a subacute or chronic disease course, and not with AKI. Rare cases of non-steroidal anti-inflammatory drug (NSAID)-induced nephrotic range proteinuria with and without AKI are recognized, but the more usual AKI provoked by NSAID exposure does not present with severely increased proteinuria [38,39]. The PUUV-induced kidney disease also differs from typical ATIN, which is rarely characterized by nephrotic range proteinuria [40]. In PUUV-induced AKI, severely increased, even nephrotic range albuminuria can be present, even with no increase in serum creatinine, as was shown in the present study.

Earlier reports about the disappearance rate of proteinuria after acute HFRS are scarce. Serial measurements of protein excretion were examined in the acute phase of Dobrava–Belgrade virus-caused HFRS in 34 patients, so that three measurements of urinary albumin, IgG and α_1-microglobulin were performed between hospital admission and 17 days thereafter [41]. The outcome was that both glomerular and tubular proteinuria declined remarkably during hospitalization, compatible with our previous finding in PUUV infection [6], but the timing in relation to the onset of fever or urinary findings after hospitalization were not reported. Cohort studies investigating urinary findings several years after acute PUUV infection show that long-lasting proteinuria is very uncommon, but some patients present with a minor amount of tubular proteinuria (urinary α_1-microglobulin) [16,42].

Albuminuria in the acute phase of PUUV infection, even when massive, disappears quickly during a period of two to three weeks after the onset of the symptoms. This type of flash-like albuminuria has not been found in other forms of AKI. We conclude that the renal manifestation of this viral infection has unique features compared to other forms of infection-associated AKI and to other proteinuric kidney diseases. Many inflammatory markers are strongly activated during PUUV infection and some of them associate with the amount of albuminuria. Both functional and structural, tubulointerstitial and glomerular changes are documented in this infection. Improved knowledge on the renal pathogenesis of this infection-related AKI could also increase our understanding about the mostly unsolved pathogenesis of other common forms of AKI. There are hardly any previous investigations about the significance of possible transient urinary findings (except the amount of diuresis) related to AKI. Although the severity of AKI in PUUV infection is associated with the amount of albuminuria in the acute phase, neither the amount of albuminuria, nor the severity of AKI, seem to affect the disappearance rate of albuminuria. When investigating the mechanisms of AKI and albuminuria in PUUV infection in the future, the exact timing of the study samples in relation to the onset of fever and albuminuria peak should be taken into consideration.

4. Materials and Methods

The study cohort consisted of 141 consecutive patients treated in Tampere University Hospital, Finland, due to acute PUUV infection, between January 2000 and December 2014. These patients also participated in our previous studies [15,43–45]. All patients provided written informed consent. The study was approved by the Ethics Committee of Tampere University Hospital (study codes 99256, R04180, R09206), and was conducted in accordance with the Declaration of Helsinki.

The median age of the patients was 42 (range 21–73) years and 96 (68%) were males. Detailed medical history was recorded and physical examination made during hospitalization. The patients had the following diagnoses before acute PUUV infection: hypertension (n = 16), asthma (n = 6), diabetes mellitus type II (n = 5), coronary artery disease (n = 5), rheumatoid arthritis (n = 4), atrial fibrillation (n = 4) inflammatory bowel disease (n = 2), epilepsy (n = 1), celiac disease (n = 1), hypothyroidism

(n = 1), sarcoidosis in remission (n = 1), history of melanoma (n = 1), polyneuropathy (n = 1), history of splenectomy (n = 1), sleep apnea (n = 1), multiple sclerosis (n = 1) and transient ischemic attack (n = 1). None of the patients had prior diagnosis of chronic kidney disease.

Acute PUUV infection was serologically confirmed in all patients either from a single serum sample by the detection of the typical granular staining pattern in the immunofluorescence assay (IFA), and/or a low avidity of IgG antibodies to PUUV, and/or PUUV IgM antibodies using an "in-house" enzyme-linked immunosorbent assay based on a recombinant antigen. The diagnostic methods have been previously described [46].

Timed overnight urine samples were collected for the determination of urinary albumin excretion (cU-Alb) once in the acute phase, once or twice in the convalescent phase 10–28 days after hospital discharge, and once at six months after the acute infection. The number of cU-Alb measurements in one patient ranged from one to three (median two).

Urine samples were conserved frozen at −70 °C. The determination of urine albumin (U-Alb) was made by an immunoturbidometric method on a Cobas C 702—Clinical chemistry analyzer (F. Hoffman—La Roche Ltd., Base, Switzerland). The detection limit of the assay for albumin was 3 mg/L. The overnight urinary albumin excretion (cU-Alb) was calculated using the volume of urine collected in a recorded timeframe (minutes) and expressed as μg/min.

Normal physiologic albuminuria was defined as cU-Alb <20 μg/min, moderately increased albuminuria as cU-Alb 20–200 μg/min, and the levels of >200 μg/min were designated as severely increased albuminuria according to the KDIGO guideline [47]. Nephrotic range proteinuria (>3 g/d) was designated as cU-Alb >1200 μg/min.

Plasma creatinine was determined using Cobas Integra (Roche), and severe AKI (stage 3) was defined according to the KDIGO definition by highest plasma creatinine >353.6 μmol/L during hospitalization [37].

Blood cell count was determined using hematological cell counters (Bayer Diagnostics, Elkhart, IN, USA), and sodium, potassium, urea, and albumin concentrations using routine automated chemistry analyzers in the Laboratory Centre of the Pirkanmaa Hospital District (later named Fimlab Laboratories), Tampere, Finland.

The statistical analyses were performed using SPSS (version 20) statistical software (IBM, Chicago, IL, USA). Medians and ranges were given for skewed continuous variables, and numbers and percentages for categorical variables.

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PUBLICATION V

High plasma resistin associates with severe acute kidney injury in Puumala hantavirus infection

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RESEARCH ARTICLE

High plasma resistin associates with severe acute kidney injury in Puumala hantavirus infection

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Abstract

Background

Puumala hantavirus (PUUV) infected patients typically suffer from acute kidney injury (AKI). Adipokines have inflammation modulating functions in acute diseases including AKI. We examined plasma levels of three adipokines (resistin, leptin, and adiponectin) in acute PUUV infection and their associations with disease severity.

Methods

This study included 79 patients hospitalized due to acute PUUV infection. Plasma resistin, leptin, adiponectin, as well as IL-6 and CRP, were measured at the acute phase, recovery phase and one year after hospitalization.

Results

Plasma resistin levels were significantly higher in the acute phase compared to the recovery phase and one year after (median resistin 28 pg/mL (11–107) vs. 17 pg/mL (7–36) vs. 14 pg/mL (7–31), p<0.001). Maximum resistin concentration correlated with maximum plasma creatinine levels (r = 0.63; p<0.001). The higher the amount of albuminuria in the urine dipstick test (0–1+, 2+ or 3+) at admission, the higher the median of maximum resistin (24.7 pg/mL, 25.4 pg/mL and 39.6 pg/mL, respectively, p = 0.002). High resistin was also an independent risk factor for severe AKI (creatinine $\geq$353.6μmol/L) (OR 1.08, 95% CI 1.02–1.14). Neither plasma leptin nor adiponectin level had any correlation with creatinine concentration or the amount of albuminuria.

Conclusions

Plasma resistin independently associates with the severity of AKI in acute PUUV infection. The association of resistin with the amount of albuminuria suggests that the level of plasma

decision to publish, or the preparation of the manuscript.

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resistin is not only influenced by renal clearance but could have some role in the pathogenesis of AKI during PUUV infection.

Introduction

Puumala virus (PUUV) belongs to the family of hantaviruses and it is spread by the bank vole (*Myodes glareolus*). In humans, PUUV causes an illness known as Nephropatia Epidemica (NE) [1]. In Finland, thousands of serologically confirmed diagnoses are made annually and the number of infected humans parallels the population of the bank vole. The disease is transmitted by inhaling dust contaminated by PUUV-infected bank vole feces or urine [2].

The characteristic manifestation of PUUV infection is a mild form of hemorrhagic fever with renal syndrome (HFRS) [3, 4]. Although many of the diagnosed patients need hospital treatment, the majority of the infections are asymptomatic or cause only mild and transient symptoms and thus remain undiagnosed [5]. In a Finnish cohort, 83% of hospital-treated patients had acute kidney injury (AKI) [6]. This syndrome has a good prognosis: kidney function returns to normal in practically all patients by supportive therapy and the mortality is low, around 0,1% [6, 7]. Several long-term nephrological, cardiovascular and endocrinological consequences have, however, been described after PUUV infection [5].

Proteinuria and hematuria are typical urinary findings in the early phase of the infection. Proteinuria is often of nephrotic range (>3g/day) and resolves rapidly. It might be a sign of change in the barrier function of the glomerular vasculature [7]. The histological finding is acute tubulointerstitial nephritis while glomerular changes are minimal [8]. Immunohistochemical studies reveal abnormalities preferentially in the tubuli and peritubular areas, where infiltrating cells consist of plasma cells, monocytes/macrophages, eosinophils and neutrophils. At the same site, the expression of tumor necrosis factor (TNF)-α as well as endothelial adhesion molecules intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 are seen, as a sign of endothelial cell activation [9].

Factors affecting the severity of PUUV infection remain mostly unclear, but host genetic factors have an influence [10]. Inflammatory markers have been shown to increase during acute PUUV infection, including plasma and urinary interleukins (ILs) IL-6, IL-1β, IL1 receptor antagonist, TNF-α, and soluble urokinase-type plasminogen activator receptor, as well as plasma pentraxin-3 and urinary gelatinase-associated lipocalin (NGAL) [11–15]. Particularly urinary IL-6 and urinary NGAL [12, 16] have been found to associate with the severity of AKI. The association of widely used C-reactive protein (CRP) with severe AKI in PUUV infection is less clear [17] [12]. We have previously found that the amount of proteinuria and hematuria in dipstick sample at the acute phase is associated with the severity of AKI in NE patients [18, 19]. The peak of proteinuria seems to precede the most severe phase of AKI [18].

Adipokines—also called adipocytokines—are bioactive molecules first found to be secreted by adipose tissue, and to regulate appetite and energy metabolism. More recently, adipokines have been discovered to be produced by many other cell types, particularly by inflammatory cells, and to regulate inflammatory responses [20]. Interestingly, plasma resistin changes have been reported in acute infections [21, 22] and AKI [23].

In the present study, our aim was to determine the adipokines adiponectin, leptin and resistin in PUUV infection, and examine their associations with the severity of acute PUUV infection and the concomitant AKI.

Materials and methods

The study cohort originally consisted of 86 consecutive patients with acute, serologically confirmed PUUV infection treated at the Tampere University Hospital in Finland, during Jan 2005-Nov 2014. Plasma samples for adipokine measurements were available in 79 patients and these patients comprised the final study cohort. Detailed medical history was obtained and physical examination was performed during the acute phase of the disease. All patients provided a written informed consent and the study was approved by the Ethics Committee of Tampere University Hospital (R04180, R09206).

Acute PUUV infection was confirmed from a single serum sample by detecting the typical granular staining pattern in immunofluorescence assay [24], and/or low avidity of IgG antibodies to PUUV [25], and/or by detecting PUUV IgM antibodies by an 'in-house' enzyme-linked immunosorbent assay (ELISA) based on a recombinant antigen [26].

Plasma samples for the measurement of resistin, leptin and adiponectin concentrations as well as CRP and IL-6 levels were collected between 7:30–8:30 am, a median of 2 (1–5) times during the acute phase. The highest or the lowest values (as appropriate) of the various variables measured during the hospital stay were designated as the maximum or minimum values. The follow-up samples were obtained a median of 15 (range 7–21) days after discharge from hospital in 74 patients, and at one year after hospitalization in 67 patients. Plasma resistin, leptin, adiponectin, CRP and IL-6 concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) using reagents from R&D Systems Europe Ltd, Abingdon, UK (resistin, leptin, adiponectin and CRP) and from eBioscience Inc, San Diego, CA, USA (IL-6). The detection limit and interassay coefficient of variation were 15.6 pg/mL and 8.5% for resistin, 15.6 pg/mL and 5.3% for leptin, 15.6 pg/mL and 6.0% for adiponectin, 3.9 pg/mL and 5.7% for CRP, and 0.39 pg/mL and 4.8% for IL-6. For adiponectin, the test detects total adiponectin.

Plasma creatinine was measured daily during hospitalization, median 5 (2–13) measurements per patient, by a Cobas Integra (F. Hoffman- La Roche Ltd., Basel, Switzerland). A urine dipstick test was performed on admission to hospital. The urine dipstick analysis was performed by automated tests based on refractometry (Siemens Clinitec Atlas or Advantus). The sensitivity of these semi-quantitative dipstick tests to urine albumin (1+) ranges 0.15–0.3 g/l. The dipstick result 2+ indicates >1 g/l albumin, and the result 3+ >3 g/l albumin. Assay for hematuria detects heme pseudoperoxidase activity and therefore it detects red cell casts and dysmorphic red cells also. The sensitivity of the assay is about 10 x 10^6 cells/L (about 3–5 cells by high power field).

Severe AKI was defined as maximum plasma creatinine level $\geq$353.6 μmol/L during hospitalization (stage 3, according to KDIGO definition) [22]. The amount of hourly urine output was not recorded. Here, shock is defined by a fall in systolic blood pressure under 90 mmHg with clinical symptoms of shock. Body mass index (BMI) was calculated as the ratio of weight (kg) to squared height (m^2).

Statistical analyses

The data is presented as medians and ranges for continuous variables and numbers and percentages for categorical variables. Groups were compared using the Mann–Whitney U test or the Kruskal-Wallis test, as appropriate. Correlations were calculated by the Spearman rank correlation test. Related samples were compared using the Wilcoxon signed rank test or the Friedman test, as appropriate. A receiver operating charasteristic (ROC) curves were drawn in order to evaluate which of the maximum levels of various inflammatory markers could act as the best indicator of severe AKI (plasma creatinine $\geq$353.6 μmol/L). A logistic regression analysis was performed with age, sex, BMI, dipstick-albuminuria class (0/1+,2+ and 3+) and

maximum plasma resistin level as independent factors to examine the association of these factors with severe AKI. Adjusted odds ratios (OR) and their 95% confidence intervals (95% CI) are given. The goodness of fit was assessed with Hosmer Lemeshow statistic. All tests were two-sided, and p-values <0.05 were considered statistically significant. The SPSS statistical software package (IBM SPSS Statistics version 23.0 Armonk, NY, USA) was used for all analyses.

Results

The clinical characteristics and laboratory findings of the patients are listed in Table 1. The median length of hospital stay was 6 days. The median age was 41 years (range 21–74) and 48 (61%) of the patients were males. The following diagnoses had been made before the acute PUUV infection in 24 (30%) patients: hypertension (n = 7), asthma/chronic obstructive pulmonary disease (n = 4), gastritis/reflux disease (n = 4), rheumatoid arthritis (n = 3), coronary artery disease (n = 2), type 2 diabetes (n = 2), type 1 diabetes (n = 1), and transient ischemic attack (n = 1). Some of the patients had more than one disease, but none had a known kidney disease or chronic renal insufficiency.

The elevation of plasma creatinine level above 100 μmol/L was detected in 56 (70%) patients. Severe AKI (creatinine $\geq$353.6 μmol/L) occurred in 25 (32%) patients. One patient needed transient dialysis treatment and two patients suffered from a clinical shock on admission to hospital. All of the patients recovered. The median of the maximum plasma creatinine at the acute phase was 186 μmol/L (range 51–1499) (Table 1). At the recovery phase (15 days after hospitalization) and one year after the acute infection, the median of creatinine was 78 μmol/L (range 55–184) and 71 μmol/L (range 53–123) respectively.

The changes in plasma adipokine, IL-6 and CRP levels in the acute phase of PUUV infection, compared to the recovery phase and one year after hospitalization, are presented in Table 2. and Fig 1. The median time to the first adipokine measurement from the onset of fever was 7 days (range 3–14). The resistin levels (Fig 1A) were significantly higher and leptin

Table 1. Clinical and laboratory findings in 79 patients hospitalized due to acute Puumala hantavirus infection.

Finding	Median	Range
Age (years)	41	21–74
Body mass index, n = 72 (kg/m^2)	26	18–37
Duration of fever (days)	8	4–15
Length of hospital stay (days)	6	2–14
Systolic blood pressure on admission (mmHg)	126	72–182
Change in body weight during hospital stay (kg)	2	0–11
Plasma creatinine max (μmol/L)	186	51–1499
Hematocrit max	0.44	0.33–0.60
Platelets min ($x10^9$/L)	52	5–150
Plasma sodium min (mmol/L)	130	109–139
Plasma potassium max (mmol/L)	4.2	3.3–5.3
Plasma albumin min (g/L)	25	18–34
Leukocytes max ($x10^9$/L)	10.8	4.2–45.0
IL-6 max (ρg/mL)	11.8	1.6–66.6
CRP max (ρg/mL)	57	8–199

Min, minimum value during hospital stay; max, maximum value during hospital stay; IL-6, Interleukin-6; CRP, C-reactive protein

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Table 2. The levels of plasma adipokines (resistin, leptin, adiponectin), and other markers of inflammation (leukocytes, plasma IL-6, CRP) in the acute phase of Puumala hantavirus infection compared to the recovery phase and one year after the infection.

	Acute phase* (n = 79)		Recovery phase (n = 74)		After 1 year (n = 67)		p-value
	median	range	median	range	median	range	
Resistin (ρg/mL)	28	11–107	17	7–36	14	7–31	<0.001
Leptin (ρg/mL)	5.3	1.2–48.4	12.2	1.6–68.7	12.1	1.8–84.0	<0.001
Adiponectin (ρg/mL)	3.76	0.23–10.66	4.07	0.62–10.25	4.36	0.80–13.49	<0.001
Leukocytes ($x10^9$/L)	10.8	4.2–44.4	7.6	3.7–14.5	6.7	3.7–11.4	<0.001
IL-6 (ρg/mL)	11.8	1.7–66.6	1.2	0.4–12.5	0.9	0.4–15.9	<0.001
CRP (ρg/mL)	57.3	8.4–198.5	1.7	0.2–36.0	1.3	0.1–13.2	<0.001

IL-6, interleukin-6; CRP, C-reactive protein. P-value stands for the differences between the three phases.

*Acute phase values are maximum (resistin, leukocytes, IL-6, CRP) or minimum (leptin, adiponectin)

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levels (Fig 1B) significantly lower in the acute phase, compared to those measured in the recovery phase or one year after the acute illness. There was also a slight but statistically significant decrease in adiponectin level in the acute phase compared to the recovery phase or one year after the acute illness (Fig 1C).

The correlations of maximum plasma resistin concentration, minimum plasma adiponectin and minimum plasma leptin with clinical and laboratory variables in the acute phase of PUUV infection are shown in Table 3. The high resistin level correlated with higher maximum plasma creatinine concentration. The median of maximum resistin was significantly higher in patients with creatinine level >100 μmol/L compared to those with maximum creatinine ≤ 100 μmol/L (resistin 32 ρg/mL, range 11–107 vs. 21 ρg/mL, range 11–42, p<0.001). The maximum resistin values also correlated with many other variables reflecting disease severity (Table 3). Interestingly, the highest measured resistin value of this cohort (107 pg/mL) was detected in one of the two patients with a clinical shock. The changes in the other two adipokines, minimum adiponectin and minimum leptin didn't have any clear correlations with the clinical disease severity markers in PUUV infection (Table 3).

The maximum plasma creatinine level measured during the hospital stay, didn't have any correlation with BMI (r = 0.145;p = 0.223). The minimum plasma leptin concentration had a weak correlation with BMI (r = 0.350; p = 0.003) but not with maximum creatinine values (Table 3).

Proteinuria on hospital admission (defined as urine albumin dipstick test ≥2+) was detected in 54 (68%) patients. Hematuria (≥2+ in urine dipstick test) was detected in 34 (43%) of the patients. When analyzing the amount of proteinuria categorized by dipstick albuminuria 0/1+, 2+ or 3+ at the acute phase, the maximum resistin level was significantly higher in patients with albuminuria 3+ than in patients with 0/1+ or 2+ albuminuria (Table 4). The other adipokines studied were not associated with dipstick-albuminuria. When combining albuminuria and hematuria findings in the urine dipstick test (0–2+, 3–4+ or 5–6+), the higher the combined positive result, the higher was the maximum plasma resistin concentration (Table 4).

We generated receiver operating curves (ROC) for the maximum plasma resistin, IL-6 and CRP levels, as well as for the maximum leukocyte count, in the acute phase to discriminate upcoming severe AKI (creatinine ≥353.6 μmol/L) from non-severe AKI (Fig 2). Resistin was a stronger discriminator with area under ROC curve (AUROC) of 0.82 (95%CI 0.70–0.91, p<0.001) when compared with leukocyte count (AUROC 0.74, 95% CI 0.62–0.87; p = 0.001). IL-6 or CRP did not discriminate severe AKI from non-severe AKI (AUROC 0.55, 95% CI 0.40–0.70; p = 0.493 and AUROC 0.39, 95% CI 0.26–0.51; p = 0.105, respectively).

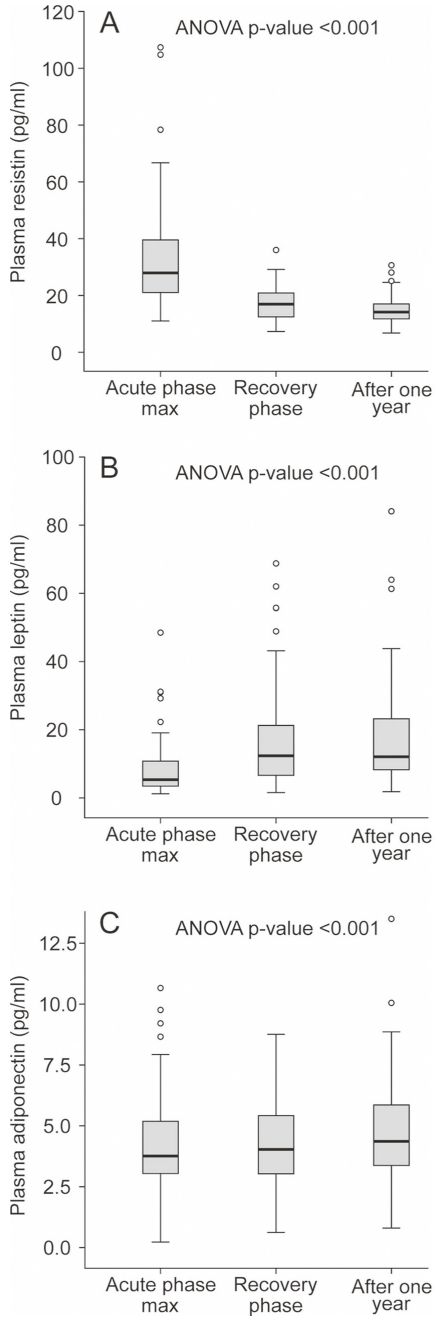
A
ANOVA p-value <0.001
Plasma resistin (pg/ml)
120
100
80
60
40
20
0
Acute phase max
Recovery phase
After one year
B
ANOVA p-value <0.001
Plasma leptin (pg/ml)
100
80
60
40
20
0
Acute phase max
Recovery phase
After one year
C
ANOVA p-value <0.001
Plasma adiponectin (pg/ml)
12.5
10.0
7.5
5.0
2.5
0.0
Acute phase max
Recovery phase
After one year

Fig 1. Plasma resistin (A), leptin (B), and adiponectin (C) levels during acute Puumala hantavirus (PUUV) infection, in the recovery phase, and one year after the hospitalization. Median (thick line inside box), 25th-75th percentiles (box), range (whiskers), and outliers (○).

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We performed a logistic regression analysis to evaluate the associations of age, gender, BMI, dipstick albuminuria and plasma resistin level with severe AKI (Table 5). In a univariate model, male gender, dipstick albuminuria of 3+, and maximum resistin level were all associated with severe AKI. In the multivariable analysis only male gender and maximum plasma resistin level were found to be independent risk factors for severe AKI.

Discussion

The present study shows that plasma resistin concentrations are elevated in the acute phase of PUUV hantavirus infection. High resistin levels correlated with the severity of AKI, as well as with several other markers reflecting the severity of the disease. High plasma resistin levels during the acute infection also associated with pronounced albuminuria and hematuria detected with urine dipstick test at hospital admission. Plasma resistin showed an independent influence on severe AKI.

Resistin was first described in 2001 when it was found to be produced by adipose tissue and promote insulin resistance in mice [27]. Further studies showed that in humans, resistin is mainly produced by leukocytes, especially macrophages, and its contribution to the development of insulin resistance remains unclear [28, 29]. The role of resistin as an inflammatory factor started to be unraveled by the early finding that injection of bacterial lipopolysaccharide (LPS) into healthy volunteers caused a rise in circulating resistin levels [30]. Specific receptors for resistin have not been identified, but it belongs to the endogenous ligands of the inflammation triggering toll-like receptor 4 (TLR-4) [31]. Accordingly, resistin is associated with an array of inflammatory diseases including sepsis, inflammatory bowel disease, arthritis and astma [32, 33]. We have previously found resistin as a contributing factor and biomarker involved in osteoarthritis [34], rheumatoid arthritis [35], inflammatory lung diseases [36, 37] and ischemia-reperfusion syndrome associated with cardiac surgery [38]. Interestingly, resistin seems also to be produced by tumors / tumor associated macrophages. For instance, resistin was recently reported as a predictive factor for the recurrence and long-term prognosis in renal cell cancer [39]. Furthermore, plasma resistin levels have been found to be significantly higher in patients with septic shock and AKI, when compared with patients with septic shock without AKI, and it was found to modulate the inflammatory response in those patients [23].

Table 3. Correlations of plasma resistin concentration with clinical and laboratory markers of disease severity in acute Puumala hantavirus infection.

	Resistin maximum, r	p-value	Adiponectin minimum, r	p-value	Leptin minimum, r	p-value
Duration of hospital stay	0.507	<0.001	0.004	0.970	0.142	0.213
Systolic blood pressure on admission	-0.257	0.022	-0.240	0.033	-0.103	0.368
Change in body weight during hospital stay	0.433	<0.001	-0.017	0.888	0.002	0.988
Creatinine max	0.633	<0.001	0.029	0.802	-0.044	0.700
Platelets min	-0.254	0.024	-0.085	0.456	0.106	0.352
Sodium min	-0.368	0.001	-0.015	0.895	0.241	0.032
Potassium max	0.369	0.001	-0.004	0.971	0.038	0.742
Leukocytes max	0.520	<0.001	0.060	0.600	-0.078	0.495
IL-6 max	0.329	0.003	-0.270	0.016	-0.160	0.160
CRP max	-0.071	0.536	-0.457	<0.001	-0.034	0.763

max, maximum; min, minimum; CRP,C-reactive protein; IL-6, interleukin-6

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Table 4. Plasma resistin levels in different categories of urine dipstick albuminuria and hematuria in 79 patients with Puumala hantavirus infection.

Urine dipstick albumin	Resistin max median (pg/mL)	range	
0–1+ (n = 25)	24.7	11.6–90.7	
2+ (n = 24)	25.4	11.9–80.4	p = 0.002
3+ (n = 30)	39.6	11.1–107.3	
Urine dipstick albumin+erythrocytes			
0–2+ (n = 26)	22.2	11.6–90.7	
3–4+ (n = 35)	27.1	11.1–80.4	p<0.001
5–6+ (n = 18)	42.7	16.8–107.3	

p-value stands for the differences between the three groups

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There are a few studies investigating the significance of resistin in other acute virus infections. Plasma resistin was elevated in the early phase of acute Dengue fever, but associations with kidney function or other disease severity markers were not reported [22]. In patients with Crimean-Congo hemorrhagic fever, caused by tick-borne virus, plasma resistin was elevated and higher concentrations associated with severe disease, defined by the manifestations of bleeding. Resistin had a negative correlation with platelet count, but it did not have a correlation with plasma creatinine. The kidney function of these patients was reported to be almost normal and AKI was not addressed [40].

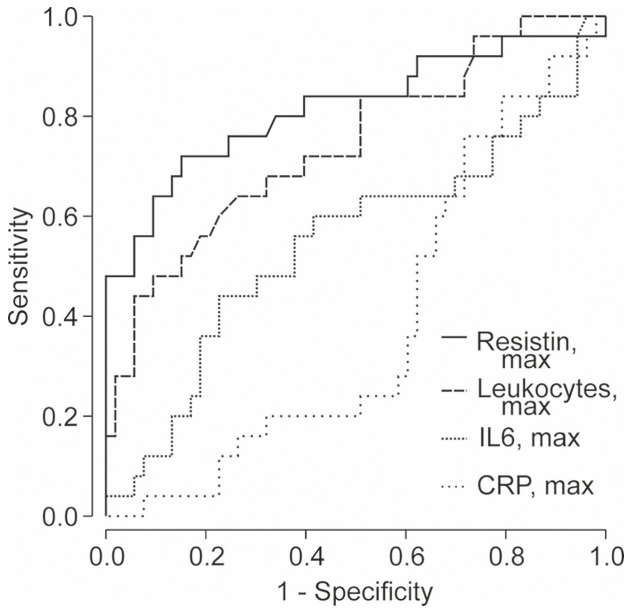

Fig 2. Receiver operating characteristic (ROC) curves for plasma resistin and different plasma inflammatory markers (maximum C-reactive protein, maximum interleukin-6, and maximum leukocyte count) in predicting severe acute kidney injury (plasma creatinine ≥353.6 μmol/L) during acute Puumala hantavirus infection.

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Table 5. Multivariate logistic regression analysis of risk factors for severe AKI (plasma creatinine ≥353.6 μmol/L) among 79 hospitalized patients with acute Puumala hantavirus infection.

			Univariate		Multivariable	
	No severe AKI n = 54	Severe AKI n = 25	OR	95% CI	OR	95% CI
	Median (range)	Median (range)				
Age (years)	42 (22–67)	39 (21–74)	0.99	0.96–1.03	1.00	0.95–1.06
Sex (n)						
Female	28	3	1		1	
Male	26	22	7.90	2.11–29.54	6.73	1.16–38.90
BMI (kg/m^2) n = 72	25.8 (18.5–37.0)	26.2 (22.6–32.3)	1.04	0.91–1.18	1.00	0.81–1.23
Albumin dipstick (n)						
0–1+	22	3	1		1	
2+	20	4	1.47	0.29–7.37	0.81	0.10–6.70
3+	12	18	11.00	2.69–45.06	4.61	0.76–28.08
Resistin max (ρg/mL)	25.0 (11.6–52.6)	45.0 (11.0–107.3)	1.10	1.05–1.154	1.08	1.02–1.14

Max = maximum, min = minimum, IL-6 = interleukin-6, CRP = C-reactive protein.

Multivariable analysis Hosmer—Lemeshow test p = 0.591

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The plasma resistin elevation in PUUV infection could be a sign of inflammation as previously shown in sepsis [21]. Plasma resistin is elevated in patients with septic shock [41, 42]. In the present study, only two patients suffered from clinical shock, one of them having the highest plasma resistin concentration of this cohort. Furthermore, high resistin level correlated with low systolic blood pressure at admission. The clinical shock syndrome in PUUV infection is related to an increased vascular permeability, which probably has a role also in the pathogenesis of the nephrotic-range proteinuria [7].

In adult intensive care unit (ICU) patients, resistin was superior to CRP in distinguishing sepsis from systemic inflammatory response (SIRS) due to trauma without infection, and the level of resistin was significantly higher in sepsis compared to trauma related SIRS [43]. In ICU patients, high resistin was shown to associate with worse long-time-survival in non-septic patients, maybe as a sign of considerable harm of excessive inflammatory response [44]. We have previously reported that plasma CRP poorly correlates with disease severity in PUUV infection compared to plasma IL-6 [12]. This finding was consolidated by the present study, where the rise in CRP did not associate with severe AKI and the ROC curves didn't show any diagnostic ability of CRP to find severe AKI. In the ROC curves maximum plasma resistin level had the best diagnostic accurancy to indicate severe AKI compared to leukocytes, IL-6 and CRP.

Many markers of inflammation are altered during acute PUUV infection, but not much is known about their relationship with proteinuria, which in turn seems to have a clear association with disease severity [18]. Increased capillary leakage seems to contribute to the amount of proteinuria during acute PUUV infection [18, 45], and some inflammation markers may have a more pronounced effect on this than others. Resistin has been previously related to vascular permeability and endothelial activation. In emergency department patients with sepsis, resistin and NGAL correlated with the expression of the endothelial cell adhesion molecules (VCAM-1, ICAM-1) [42] and were associated with septic shock, but not with mortality [41]. Any possible association with AKI was not discussed.

Plasma levels of adiponectin did not show any association with maximum creatinine values or albuminuria in hospital-treated patients in the present study. Low plasma adiponectin had, however, negative correlation to inflammatory markers CRP and IL-6. Adiponectin is a

multifunctional cytokine with mainly anti-inflammatory properties [20]. Among adipokines, adiponectin is the most abundant in human serum. Elevated adiponectin concentrations are seen in many inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease, as well as in chronic kidney disease (CKD) [20]. Adiponectin has functions protective against obesity-related diseases but its concentration is decreased in obesity. The view of a possible role of adiponectin in AKI is mainly based on animal studies [46]. A protective effect of adiponectin has been shown in a murine model of ischemia-reperfusion -induced AKI [47]. If adiponectin has any protective effect on the development of AKI in humans, remains unclear.

Leptin deficient mice are susceptible to severe AKI caused by lipopolysaccharide induced endotoxic shock [48]. In the present study, leptin levels were decreased at the acute phase of PUUV infection, but even after adjustment by BMI, low leptin level did not correlate with the severity of AKI. When plasma leptin levels were compared between CKD patients on hemodialysis, patients with AKI, and healthy subjects, the patients with AKI were shown to have similar leptin levels as healthy subjects, while the level was clearly elevated in hemodialysis patients [49]. After kidney transplantation, the level of plasma leptin is shown to decline already during the first day [50]. Although kidneys are considered as the main site of leptin metabolism, the alterations of leptin level are possibly influenced by many coexisting factors affecting both its synthesis and degradation [49]. Circulating leptin level is highly correlated with fat mass and leptin concentrations have been reported to decrease in healthy subjects following fasting [51]. Anorexia, nausea and abdominal pain, and associated reduced food intake, frequently reported symptoms in NE, could partly explain low acute-phase leptin levels in the present study. It can't be excluded, however, that low leptin at the acute phase of PUUV infection may contribute to the development of AKI.

There are scarce previous data about adipokine levels in patients with AKI. It is possible that the rise in plasma resistin level reflects accumulation of that particular adipokine during reduced glomerular filtration rate (GFR), since plasma resistin concentrations have been found to be elevated in uremia [52]. The molecular weight of resistin is relatively low 12,5 kDa compared to leptin 16 kDa and albumin 66 kDa. The molecular weight of adiponectin different multimers are 180–360 kDa. We believe that decreased GFR but also an increased synthesis of resistin can influence the elevation of plasma resistin level. In the present study, resistin had strong association with proteinuria and hematuria. In the multivariate analysis high resistin level remained (along with male gender) as an independent risk factor while the high amount of dipstick albuminuria (3 +) did not. To summarize, the observed rise in plasma resistin level may not be explained by mere accumulation, but also increased synthesis, and thus resistin might have an independent influence on the development of AKI. As a limitation, we were not able to determine the point of time after the onset of fever when resistin peaks, because of varying delay of patient admission. The time course between resistin peak and the most severe AKI phase remains to be investigated.

In conclusion, plasma resistin concentration is elevated in acute PUUV hantavirus infection and it declines early during the recovery phase. Plasma resistin level is related to the severity of AKI, as well as to the amount of proteinuria and hematuria. An increased plasma resistin concentration was found to be an independent risk factor for severe AKI in PUUV infected patients also when adjusted to dipstick albuminuria. Further studies are needed to understand the role of resistin as an AKI biomarker or possible treatment target in patients with PUUV infection.

Supporting information

S1 Dataset. The complete data used in the analysis is in the excel file appendix.
(XLSX)

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