Tampere University

LAURA TERVO

Factors Associated with the Clinical Severity of Puumala Hantavirus Infection

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ACADEMIC DISSERTATION
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To Antti, Otto and Emil

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Tampere, March 2023

Laura Tervo

ABSTRACT

Hemorrhagic fever with renal syndrome (HFRS), caused by Puumala hantavirus (PUUV), also called nephropathia epidemica, is a common febrile illness in Finland. PUUV infection begins with a sudden high fever accompanied by headache, vomiting, abdominal and back pain, and visual disturbances. Renal involvement comprises the oliguric phase followed by polyuria and usually spontaneous recovery. Acute kidney injury (AKI) is severe in one-third of hospitalized patients. Nevertheless, the outcome of PUUV infection is usually favorable, and the case fatality rate is <1%.

Cigarette smoking and alcohol consumption have been shown to affect the course of several infections. Smoking leads to structural and functional changes in the airways. In addition, smoking alters the immune system in several ways, affecting the susceptibility to acquire infection, and the clinical course of some infections. Furthermore, alcohol consumption affects the susceptibility to and course of some infections.

This study was carried out to investigate the possible effects of these two life-style factors on the clinical course of PUUV infection. It also aimed to examine abdominal fluid collections during the acute phase of PUUV infection by means of magnetic resonance imaging (MRI), and the role of a biomarker, soluble urokinase-type plasminogen activator receptor (suPAR), as a predictor of the clinical severity of PUUV infection.

A history of smoking among 357 patients was collected via a questionnaire. Cigarette smoking predisposed patients to more severe AKI, and current smokers had higher maximum serum creatinine levels than nonsmokers. A severe AKI, defined as serum creatinine level ≥ 353.6 μmol/L, was more prevalent in smokers than in nonsmokers. Smoking cessation returned the risk of severe AKI to the same level as in never-smokers.

Alcohol consumption was studied by detecting biomarkers showing previous alcohol use in 66 patients. A combination marker, GGT-CDT, can detect heavy alcohol consumption with a sensitivity of 90% and specificity of 98%. The other measured biomarker was urinary ethyl glucuronide (EtG), which detects recent alcohol consumption. Altogether, 41% of the patients showed biochemical signs of

recent alcohol consumption at the control visit. Nevertheless, alcohol use did not seem to predispose patients to more severe PUUV infection. The levels of liver enzymes and serum amylase were also evaluated. Liver enzymes were slightly elevated during acute infection but were not associated with more severe infection. No cases of acute pancreatitis were found.

The hallmark of the pathogenesis of hantaviral diseases is increased endothelial permeability with capillary leakage. Pleural fluid is often reported during PUUV infection, but the presence of abdominal fluid collections is far less well studied. The present study was aimed at investigating abdominal fluid below the diaphragm and the amount of pleural fluid by means of MRI in 27 PUUV-infected patients. Fluid collections were assessed in relation to the patients' symptoms and clinical and laboratory findings. All patients showed additional fluid. Fluid was detected most frequently in the perirenal space, next to the psoas muscle, and in the pouch of Douglas. Pleural fluid was found in 25 of the patients. Patients with more severe fluid collections did not have symptoms more often than patients with lesser amounts of fluid. The amounts of both intraperitoneal and retroperitoneal fluid correlated inversely with serum creatinine levels. The amount of retroperitoneal fluid also showed an inverse correlation with serum cystatin C concentrations. Increased intraperitoneal fluid was also associated with higher C-reactive protein (CRP) concentration.

Several biomarkers have been shown to predict disease severity in infectious diseases. SuPAR is a multifunctional glycoprotein the concentrations of which are elevated in various inflammatory and infectious conditions. SuPAR levels were measured in 97 patients in the acute phase of PUUV infection and in the convalescence phase. SuPAR concentrations were significantly higher in the acute phase compared with the convalescence phase. Higher concentrations were associated with more severe AKI. Plasma suPAR levels correlated with serum creatinine concentrations, and patients who needed dialysis treatment had higher suPAR concentrations than patients who did not need dialysis.

In conclusion, cigarette smoking leads to more severe AKI in hospitalized patients with PUUV infection. On the other hand, alcohol consumption does not seem to affect the course of the infection. Levels of liver enzymes are often elevated but are not associated with disease severity. In abdominal MRI, fluid collections are present in hospitalized PUUV-infected patients. More prominent fluid collections were not associated with more severe AKI. In fact, more prominent fluid may present as a protective sign against severe AKI. When evaluating patients with

biomarkers, plasma suPAR concentrations were associated with more severe AKI in PUUV-infected patients.

TIIVISTELMÄ

Puumala-hantaviruksen (PUUV) aiheuttama myyräkuume on Suomessa yleisesti esiintyvä infektiotauti. Se alkaa äkillisellä korkealla kuumeella, johon liittyy päänsärkyä, oksentelua, vatsa- ja selkäkipua sekä näköhäiriöitä. Munuaistautiin kuuluu oligurinen vaihe, jota seuraa polyuria ja yleensä spontaani toipuminen. Akuutti munuaisvaurio (acute kidney injury, AKI) on vakava noin kolmasosalla sairaalahoitoa vaatineista potilaista. Myyräkuumeen taudinkuva on kuitenkin yleensä suotuisa ja kuolleisuus tautiin on vähäinen, alle 1 %.

Tupakoinnin ja alkoholin käytön on osoitettu vaikuttavan infektiosairauksien taudinkuvaan. Tupakointi johtaa rakenteellisiin ja toiminnallisiin muutoksiin hengitysteissä. Lisäksi tupakointi vaikuttaa immuunijärjestelmään monin tavoin. Se johtaa infektioalttiuteen ja vaikuttaa myös joidenkin infektioiden kliiniseen taudinkuvaan. Myös alkoholi vaikuttaa infektioalttiuteen ja joidenkin infektioiden taudinkulkuun.

Tässä väitöskirjatyössä selvitettiin näiden kahden elämäntapatekijän mahdollista vaikutusta myyräkuumeen taudinkuvaan. Lisäksi selvitettiin, onko magneettitutkimuksella todettavissa nestekertymiä ja näiden mahdollista yhteyttä taudinkuvaan. Tutkimuksessa selvitettiin myös liukoisen urokinaasityyppisen plasminogeenia aktivoivan reseptorin (suPAR) roolia myyräkuumeen vaikeusasteen ennustamisessa.

Tupakointihistoria selvitettiin 357 myyräkuumepotilaalta kyselylomakkeella. Tupakointi altisti potilaan vaikeammalle AKI:lle, tupakoitsijoilla oli korkeampi seerumin kreatiniinipitoisuus kuin tupakoimattomilla. Vaikea AKI, jonka määritelmänä oli seerumin kreatiniinipitoisuus ≥ 353.6 μmol/L, oli yleisempi tupakoitsijoilla kuin tupakoimattomilla. Tupakoinnin lopettaminen palautti kuitenkin vakavan AKI:n riskin tupakoimattomien tasolle.

Edeltävää alkoholin käyttöä tutkittiin biologisilla merkkiaineilla 66 potilaalla. Yhdistelmämerkkiaine GGT-CDT:llä voidaan tunnistaa runsas alkoholinkäyttö 90 %:n herkkyydellä ja 98 %:n tarkkuudella. Toinen mitattu merkkiaine oli virtsan etyyliglukuronidi (EtG), jolla voidaan havaita hiljattainen alkoholinkäyttö. Yhteensä 41 prosentilla potilaista havaittiin kontrollikäynnillä biokemiallisia merkkejä viimeaikaisesta alkoholinkäytöstä. Alkoholin käyttö ei kuitenkaan näyttänyt altistavan

potilaita vaikeammalle myyräkuumeelle. Tässä tutkimuksessa määritettiin myös seerumin maksaentsyymit ja amylaasi. Maksaentsyymit olivat hieman koholla akuutin infektion aikana, mutta niiden nousu ei liittynyt vakavampaan infektioon. Akuutteja haimatulehduksia ei havaittu.

Hantavirusinfektioiden patogeneesissä on keskeistä lisääntynyt endoteelin läpäisevyys ja kapillaarivuoto. Pleuranesteen kertymistä on raportoitu PUUV-infektiossa, mutta vatsan alueen nestekertymiä on tutkittu varsin vähän. Tässä tutkimuksessa selvitettiin magneettikuvauksella (MRI) pallean alapuolisia nestekertymiä sekä pleuranestekertymiä 27 PUUV-infektioon sairastuneella potilaalla. Nestemäärää verrattiin potilaiden oireisiin, sekä kliinisiin- että laboratoriolöydöksiin. Kaikilla potilailla todettiin ylimääräistä nestettä. Sitä havaittiin yleisimmin perirenaalitilassa, psoas-lihaksen vieressä, ja fossa Douglasissa. Pleuranestettä havaittiin 25 potilaalla. Potilaat, joilla oli suuremmat nestekertymät, eivät kuitenkaan oireilleet useammin kuin potilaat, joilla oli vähäisempi nesteretentio. Sekä intra- että retroperitoneaalisen nesteen määrä korreloi käänteisesti seerumin kreatiniinipitoisuuden kanssa. Retroperitoneaalisen nesteen määrällä oli käänteinen korrelaatio myös seerumin kystatiini C-pitoisuuksien kanssa. Lisääntynyt nesteen kertyminen intraperitoneaalitilaan liittyi korkeampiin C-reaktiivisen proteiinin (CRP) pitoisuuksiin.

Useiden biologisten merkkiaineiden on osoitettu pystyvän ennustamaan infektiotaudin vakavuutta. Liukoinen urokinaasityyppinen plasminogeenia aktivoiva reseptori (suPAR) on glykoproteiini, jonka pitoisuus lisääntyy erilaisissa tulehdustiloissa ja infektioissa. Plasman suPAR mitattiin 97 potilaalta akuutin PUUV-infektion aikana ja toipilasvaiheessa. SuPAR-pitoisuudet olivat akuutissa vaiheessa merkitsevästi suuremmat kuin toipilasvaiheessa. Korkeammat pitoisuudet liittyivät vaikeampaan AKI:iin. Plasman suPAR-pitoisuudet korreloivat seerumin kreatiniinipitoisuuksien kanssa, ja dialyysihoitoa tarvitsevilla potilailla havaittiin korkeammat suPAR-pitoisuudet kuin potilailla, jotka eivät tarvinneet dialyysihoitoa.

Yhteenvetona voidaan todeta, että tupakointi johtaa vaikeampaan akuuttiin munuaisvaurioon sairaalahoitoon joutuneilla myyräkuumepotilailla. Sen sijaan alkoholin käyttö ei näytä vaikuttavan myyräkuumeen taudinkuvaan. Maksaentsyymien pitoisuus lisääntyy akuutissa myyräkuumeessa, mutta ilmiö ei liity vaikeampaan tautiin. Vatsan MRI-tutkimuksessa nähdään nestekertymiä sairaalahoitoa vaatineilla myyräkuumepotilailla, mutta suuremmat nestekertymät eivät liity vaikeampaan akuuttiin munuaisvaurioon. Sen sijaan nestekertymät voivat näyttäytyä munuaisvaurion kannalta suojaavana tekijänä. Kun arvioidaan potilaita

biologisilla merkkiaineilla, plasman suPAR-pitoisuus on koholla akuutissa myyräkuumeessa ja se korreloi akuutin munuaistaudin vaikeuteen.

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ABBREVIATIONS

AKI	acute kidney injury
ALT	alanine aminotransferase
AM	alveolar macrophage
AMYL	amylase
C3	complement component 3
CCHF	Crimean-Congo hemorrhagic fever
CDT	carbohydrate-deficient transferrin
cf-DNA	cell-free deoxyribonucleic acid
CI	confidence interval
CKD	chronic kidney disease
CNS	central nervous system
COVID-19	coronavirus disease 2019
CRP	C-reactive protein
CS	cigarette smoke
Cys C	cystatin C
DOBV	Dobrava virus
ELISA	enzyme-linked immunosorbent assay
EtG	ethyl glucuronide
FSGS	focal segmental glomerulosclerosis
GGT	gamma-glutamyl transferase
HCPS	hantavirus cardiopulmonary syndrome
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRCT	high-resolution computed tomography
HTNV	Hantaan virus
IDO	indoleamine 2,3-dioxygenase
Ig	immunoglobulin
IL	interleukin
INF	interferon

IRR	incidence rate ratio
KDIGO	Kidney disease: Improving global outcomes
MRI	magnetic resonance imaging
NE	Nephropathia epidemica
NET	neutrophil extracellular trap
NK	natural killer
NO	nitric oxide
OR	odds ratio
PCT	procalcitonin
PEth	phosphatidylethanol
PTX-3	Pentraxin-3
PUUV	Puumala virus
ROS	reactive oxygen species
SAAV	Saarenmaa virus
SC5b-9	membrane attack complex of the complement system
SEOV	Seoul virus
SNV	Sin Nombre virus
suPAR	soluble form of urokinase-type plasminogen activator receptor
TGF-β1	transforming growth factor-β1
TNF	tumor necrosis factor
UAER	urinary albumin excretion rate
uPAR	urokinase-type plasminogen activator receptor
US	ultrasound
U-Trypsin	urinary trypsinogen
VEGF	vascular endothelial growth factor

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-IV. In addition, some unpublished data are presented.

I Tervo L, Mäkelä S, Syrjänen J, Huttunen R, Rimpelä A, Huhtala H, Vapalahti O, Vaheri A, Mustonen J. Smoking is associated with aggravated kidney injury in Puumala hantavirus-induced haemorrhagic fever with renal syndrome. Nephrology, Dialysis, Transplantation 2015;30(10):1693-8. http://doi:10.1093/ndt/gfv273.

II Tervo L, Outinen TK, Mäkelä S, Mustalahti J, Huhtala H, Pörsti I, Syrjänen J, Mustonen JT, Niemelä O. Alcohol consumption and its influence on the clinical picture of Puumala hantavirus infection. Viruses 2022; 14(3):500. http://doi:10.3390/v14030500

III Tervo L, Outinen T, Kiekara T, Tietäväinen J, Paakkala A, Pörsti I, Huhtala H, Mäkelä S, Mustonen J. The presence of intraperitoneal, retroperitoneal and pleural fluid in acute Puumala hantavirus infection. Infectious Diseases (London) 2022; Dec 23; 1-9. http://doi: 10.1080/23744235.2022.2160010.

IV Outinen TK, Tervo L, Mäkelä S, Huttunen R, Mäenpää N, Huhtala H, Vaheri A, Mustonen J, Aittoniemi J. Plasma levels of soluble urokinase-type plasminogen activator receptor associate with the clinical severity of acute Puumala hantavirus infection. PlosOne 2013; 21;8(8): e71335. http://doi: 10.1371/journal.pone.0071335.

AUTHOR'S CONTRIBUTION

Studies I-III: The author designed the studies together with supervisors and other study-group members. In Study I, the author designed the questionnaire with the help of co-authors. The author conducted a literature search. The author analyzed the data with the support of the statistician and wrote the first drafts and was the first author of these studies. The author submitted the manuscripts to the journals and provided the final versions of the papers.

Study IV: The author contributed to preparing the manuscript but did not perform the analysis of data in Study IV.

1 INTRODUCTION

Puumala virus (PUUV) belongs to the orthohantaviridae family and is the most common hantavirus in Europe. Each hantavirus has its own carrier animal, and PUUV is carried by the bank vole (*Myodes glareolus*). PUUV causes a mild form of hemorrhagic fever with renal syndrome (HFRS). It is highly endemic in Finland, with approximately 1000–3000 serologically verified cases annually [1]. The seroprevalence in Finland is higher than the incidence rate, recently estimated as 12.5% [2]. The virus is transmitted to humans by inhaling infected dust containing the rodents' excreta contaminated by the virus. Predisposing factors for PUUV infection are living in a house with holes for rodents to enter the building, handling firewood, and cigarette smoking [2–4].

The clinical picture of PUUV infections varies. Five phases determine the course of the infection: febrile, oliguric, hypotensive, polyuric, and convalescence phases. Since the natural course of PUUV infection is usually mild, these phases are not always clear. Usually, the disease begins with a sudden high fever. Gastrointestinal symptoms, such as nausea, vomiting, and abdominal pain, may follow. Back pain has been reported to present in 33–82% of the patients [5–9]. Typical laboratory findings are low platelet count, elevated creatinine, and C-reactive protein (CRP) concentrations, and leukocytosis [10]. Elevated levels of liver enzymes are often reported during acute PUUV infection [11], but their clinical relevance and association with the severity of PUUV infection remain unelucidated. Renal involvement presents as transient, often oliguric acute kidney injury (AKI) with microscopic hematuria and proteinuria. The most common renal histopathological finding is acute tubulointerstitial nephritis with medullary hemorrhages in 20–60% of the biopsies [12,13].

In addition to AKI and thrombocytopenia, increased capillary leakage is another hallmark of PUUV infection. It can result in fluid accumulations, hypotension, and even shock [14]. Only a few studies have been carried out to explore the accumulation of fluid in the abdomen during acute PUUV infection [15–17].

Cigarette smoking has adverse effects on human health. Cigarette smoking affects the immune system in various ways and predisposes individuals to many infectious

diseases [18]. Smoking also results in worsened outcomes in several infectious diseases [19]. There are previous reports of smoking as a predisposing factor for acquiring PUUV infection [20,21], but there are no previous reports on the association between cigarette smoking and disease severity.

Alcohol consumption and cigarette smoking have been shown to be associated in several studies [22–24]. Furthermore, chronic use of alcohol influences the human immune system and predisposes individuals to some infections. Alcohol consumption may also worsen the clinical outcome of infections [25,26]. Chronic alcohol consumption can be measured by way of several markers, of which gamma-glutamyl transferase (GGT) and carbohydrate-deficient transferrin (CDT) are widely used. A combination marker of GGT-CDT has been developed to increase specificity without losing sensitivity as compared with conventional markers [27].

Several biomarkers have been shown to predict the severity of PUUV infection [28–31]. Plasma soluble urokinase receptor (suPAR) is a multifunctional glycoprotein, the levels of which have been shown to be elevated during various inflammatory and infectious states [32]. This novel biomarker has not been previously studied in hantaviral diseases.

The aim of this thesis was to study whether cigarette smoking and/or alcohol consumption alter the clinical course of acute PUUV infection. A second aim was to elucidate whether commonly found elevated liver enzymes are associated with the severity of acute PUUV infection. A third aim was to evaluate the amounts and distribution of fluid collections in the abdomen and pleural space during acute PUUV infection and to evaluate suPAR concentrations during acute illness and their association with disease severity.

2 REVIEW OF THE LITERATURE

2.1 Puumala virus and other hantaviruses

2.1.1 Virology

Puumala virus (PUUV) belongs to the Bunyavirales group of the Hantaviridae family [33]. It is an enveloped single-stranded RNA virus. The diameter of hantaviruses ranges from 80 to 120 nm [34]. Hantaviruses have a three-segmented genome, in which the large segment encodes viral RNA-dependent RNA polymerase (L protein), transcripting and replicating the viral genome. The medium segment encodes enveloped glycoproteins Gn and Gc, and the small segment encodes nucleocapsid (N) protein, which encapsulates the genomic RNA into three viral chromosomes [34,35]. Hantaviruses can infect epithelial, endothelial, macrophage, follicular dendritic, and lymphocyte cells [34,36]. The primary receptor of pathogenic hantaviruses for cell-entry is β3-integrin, a transmembrane protein that promotes cell-cell adhesion [37]. Virus replication and budding take place in the Golgi apparatus or nearby, and the virions are released via exocytosis to infect new cells [37].

Each hantavirus has its specific carrier host. PUUV is carried by the bank vole (*Myodes glareolus*), which is found all over Europe, except on the Mediterranean coast, most of Spain and Portugal, part of Ireland and Northern Ireland and tundra parts of Finland, Sweden, and Norway [11]. Carrier animals are chronically and usually asymptomatically infected, and they excrete the virus in their urine, feces, and saliva [11]. Humans get the infection by inhaling contaminated aerosolized excreta. Hantaviruses cause two kinds of syndrome, hemorrhagic fever with renal syndrome (HFRS) in Europe, Asia, and Russia, and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. Viruses causing HFRS have been called old-world hantaviruses, the disease being first described in China 900 years ago [36]. HCPS-causing new-world hantaviruses were first recognized in 1993 in America when a large outbreak of pulmonary distress syndrome emerged. The causative agent was named Sin Nombre virus (SNV), and soon after, many other new hantaviruses were

isolated [36]. The two hantavirus syndromes share the same pathogenesis even though the clinical outcome is different. Therefore, these syndromes have been proposed to be called Hantaviral disease and Hantavirus fever [38]. Table 1 shows human pathogenetic hantaviruses and their carrier hosts. Only Andes virus has been shown to transmit from human to human [39].

Table 1. Pathogenic hantaviruses and their carrier animals.

HFRS				HCPS		
Europe		Carrier animal		Americas		Carrier animal
PUUV	Puumala virus	Myodes glareolus/ Bank vole		ANAJV	Anajatuba virus	Oligoryzomys fornesi/ Fornes' colilargo
DOBV	Dobrava virus	Apodemus flavicollis/ Yellow-necked mouse		ANDV	Andes viruses	Oligoryzomys longicaudatus/ Long-tailed mouse
SAAV	Saarenmaa virus	Apodemus agrarius/ Striped field mouse		ArQV	Araraquara virus	Bolomys lasiurus/ Hairy-tailed Bolo mouse
SEOV	Seoul virus	Rattus norvegicus/ Brown rat		BAYV	Bayou virus	Oryzomys palustris/ Rice rat
TULV	Tula virus	Microtus arvalis/ Common vole				
				BMJV	Bermejo virus	Bolomys lasiurus/ Hairy-tailed Bolo mouse
Asia				BCC	Black Creek Canal virus	Sigmodon hispidus/ Cotton rat
HTNV	Hantaan virus	Apodemus agrarius/ Striped field mouse		CASV	Castelo dos sonhos	Oligoryzomys eliurus/ Brasilian pygmy rice rat
AMRV/SOOV	Amur/Soochong virus	Apodemus peninsulae/ Korean field mouse		CHOV	Choclo virus	Oligoryzomys fulvescens/ Pygmy rice rat
SEOV	Seoul virus	Rattus norvegicus/ Brown rat		LANV	Laguna Negra virus	Calomys laucha/ Vesper mouse
THAIV	Thailand virus	Bandicota indica/ Greater bandicoot rat		LECHV	Lechiguanas virus	Oligoryzomys flavescens/ Yellow pigmy rice rat
LUXV	Luxi virus	Eothenomys miletus/ Large Chinese vole		NY-1	New York virus	Peromyscus leucopus/ White-footed mouse
				ORNV	Oran virus	Oligoryzomys longicaudatus/ Long-tailed mouse
Africa				SNV	Sin Nombre virus	Peromyscus maniculatus/ Deer mouse
SANGV	Sangassou virus	Hylomyscus simus/ African wood mouse				

2.1.2 Epidemiology

Globally, 150,000–200,000 cases of HFRS occur annually. Most cases are caused by Hantaan virus (HNTV) in China [34,40]. PUUV causes the majority of HFRS cases in Europe, followed by Dobrava virus (DOBV) and Saarenmaa virus (SAAV) [11,36]. PUUV was first detected in the lung of a bank vole in 1977 and the virus was named according to the place the rodent was caught [41,42]. Nephropathia epidemica (NE), the febrile illness due to PUUV infection, was nevertheless described for the first time in 1934, in north-central Sweden [9]. In modern literature, "PUUV infection" has replaced the term "Nephropathia epidemica" and is used in this thesis.

The incidence of PUUV infection follows the cycle of the bank vole population. Lately, climate change has altered earlier patterns. In Central Europe, i.e., Germany and Belgium, the incidence of PUUV is growing [43]. The abundance of bank voles has been related to the high seed production of oak and beech. Recently, the previous 3-year cycle of outbreaks in Belgium has turned out to occur every second year [43]. In Finland, earlier high-peak years every three to four years have turned towards annual fluctuations [43]. The epidemic in Finland usually appears in one or two peaks between August and January, and the incidence is lowest during spring.

According to the Finnish Institute for Health and Welfare [1], the last high peak year in Finland was 2014 [1]. It has been speculated that the incidence in the northern parts of Europe will decrease over the long term due to milder and wetter winters, leading to loss of protective snow shelter for rodents [43]. In contrast, a large PUUV outbreak, with an incidence of 300 cases/100 000 persons, was reported in Västerbotten, Sweden, in 2007 when the winter was unusually warm, and the rodents entered buildings looking for shelter [44].

The incidence in Finland is, on average, 31–39 cases/100 000 persons [2,45,46]. This is an underestimate of the actual number of cases, because the majority of cases are mild and do not warrant medical contact, thereby leading to a lack of serologically verified diagnoses. The seroprevalence of 12.5% in the Finnish population is the highest reported worldwide [2]. In a recent Finnish study the estimated notified/total PUUV infection incidence ratio was reported to be 1:11 [2]. In northern parts of Sweden even higher prevalence numbers, up to 13.4%, have locally been reported [47].

Farming, handling firewood and rodent traps, and cigarette smoking have been shown to predispose individuals to PUUV infection in earlier studies [2,3,21,48]. In two studies, smoking has shown considerable odds ratios (ORs) of 9.1 and 3.6 as

independent risk factors of PUUV infection [20,21]. Finnish and Swedish seroprevalence studies have confirmed the association between smoking and PUUV seropositivity [2,49]. In addition, PUUV infection is more prevalent in males than in females. In a large registry-based study of 22 681 PUUV-infected patients, 62% of the cases were males [50].

2.2 Puumala virus infection

2.2.1 Clinical and histopathological features

The clinical picture of PUUV infection varies from subclinical to fatal, though the typical course of the infection is relatively mild. The fatality rate in PUUV infection is low, 0.08–0.4% [46,51]. The infection presents in five phases: febrile, hypotensive, oliguric, polyuric and convalescent phases [11,36]. Considering that PUUV infection is usually milder than, for example, DOBV infection, these phases are not always clearly distinguishable. Symptoms appear after 2–8 weeks of incubation [6]. The disease begins with a high fever accompanied by a headache. These are typically followed by gastrointestinal symptoms, such as nausea, vomiting, diarrhea, and abdominal pain, which are present in 30–60% of hospitalized patients [5–9]. Back pain is present in 33–82% of patients during the acute phase of PUUV infection [5–9]. Ocular symptoms are common in PUUV infection, with a prevalence of 33–70% among hospitalized patients [52,53]. A myopic shift is proposed to result from thickening of the lens [52]. In addition, other central nervous system (CNS) symptoms, such as headache, insomnia, dizziness, and somnolence, are often present. In a Finnish study of 58 hospitalized PUUV-infected patients, 87% of the participants suffered from symptoms indicating CNS involvement [54].

Severe bleeding complications are rare, but mild hemorrhages occur. Epistaxis, conjunctival bleedings, petechiae, macroscopic hematuria, and gastrointestinal bleedings have been reported [6,7,55]. Panhypopituitarism has been described in case reports and small series, and at least partly, it is caused by hemorrhages and necrosis of the pituitary gland [56–62].

Kidney disease in PUUV infection includes a transient oliguric phase followed by polyuria and spontaneous recovery. A characteristic histopathological finding in the kidney is acute tubulointerstitial nephritis with infiltrating lymphocytes, plasma cells, monocytes, macrophages, and polymorphonuclear leukocytes [12,13,63]. Of

lymphocytes, CD8+ cells are predominant [63]. A French study showed acute tubular necrosis in fifteen out of seventeen patients [64]. The study also revealed medullary hemorrhages in 9/10 patients, but in only 2/7 of the "cortex-only" samples [64]. In earlier studies, medullary hemorrhages have been found in 20–60% of the biopsies [12,13].

2.2.2 Laboratory findings

The most characteristic laboratory finding in acute PUUV infection is thrombocytopenia, seen in 52–90% of patients [6–9,65]. In a Finnish cohort, thrombocytopenia was present in 90% of 546 patients, and 28% of the patients' platelet counts fell below 50×10^9/L [65]. In that study, thrombocytopenia was associated with variables reflecting capillary leakage and inflammation but not with the severity of AKI [65]. French and German studies, however, have shown an association between low platelet count and more severe AKI [66,67]. The mechanism of thrombocytopenia is not fully understood, but an interaction between thrombocytes and infected endothelium contributes to the decreased number of circulating thrombocytes [68]. Furthermore, the consumption of thrombocytes may result from enhanced platelet adhesion and activation [69].

AKI is another hallmark of acute PUUV infection. Elevation of creatinine concentrations is seen in the majority of hospitalized patients. In a Finnish study of 126 patients, the mean creatinine level was 439 μmol/L, and renal function was transiently impaired in 94% of the patients [7]. Electrolyte changes are common during acute PUUV infection. Hypocalcemia was seen in 89%, hyponatremia in 58%, and hypokalemia in 31% of the patients [7]. Despite the high frequency of AKI, hyperkalemia was seen rarely, in only 12% of the patients, and only one patient showed a serum potassium level of over six mmol/L [7]. Acute-phase renal involvement appears as proteinuria, which can be found in up to 80–94% of patients [8,70]. Proteinuria can be massive, and it is nonselective [70]. Hematuria is also commonly present in the acute phase of PUUV infection. Microscopic hematuria has been reported in 60–90%, and macroscopic hematuria in 7% of cases [6,7,9,71]. In addition, glucosuria has been reported, but not as often as proteinuria or hematuria [72]. All these urinary findings can predict the severity of PUUV infection [71–73].

Levels of liver enzymes are typically elevated during acute PUUV infection. Raised levels of serum aminotransferases have been reported in 41–85% of patients

[6,11,74,75], but the association between elevated aminotransferases and the clinical severity of acute PUUV infection remains unknown. There is only one report on PUUV-induced acute pancreatitis [60]. Instead, a German study showed elevated serum lipase concentrations in 15% of hospitalized patients, but no signs of acute pancreatitis by means of ultrasound or computed tomography (CT) imaging [76]. Serum amylase levels during acute PUUV infection are not reported in general.

The third hallmark of acute PUUV infection is increased capillary leakage, which can be measured only indirectly by way of laboratory markers. High hemoconcentrations are seen as high hematocrit and hemoglobin levels when increased capillary permeability occurs. Increased capillary leakage may lead to hypotension and even shock. The pathogenesis and possible mechanism of capillary leakage are discussed in detail in Section 2.3. Leukocyte levels are often elevated, and leukocytosis reflects the inflammation stage. Plasma CRP concentrations are elevated in almost all patients during acute PUUV infection[10].

2.2.3 Biomarkers predicting the severity in Puumala virus infection

Several biomarkers can predict the severity of acute PUUV infection. A multifunctional cytokine, interleukin-6 (IL-6), is produced during infection and inflammation. In PUUV patients, IL-6 production increases during the acute phase, and high plasma levels are associated with the severity of PUUV infection measured by means of several variables, including more severe AKI [10]. CRP is a widely used biomarker of infection and inflammation. In addition, CRP levels are elevated in various other conditions, such as trauma, malignancies, and myocardial infarction [10]. The main functions of CRP are activation of the classical complement pathway, induction of cytokine production, and enhancement of opsonin-mediated phagocytosis [10]. The ability of CRP to predict the severity of PUUV infection is controversial. In a Finnish study of 118 PUUV-infected patients, CRP levels were not associated with thrombocytopenia, leukocytosis, or length of hospital stay. Neither did CRP levels correlate with more severe AKI. Conversely, CRP was shown to have a protective role against kidney injury in that study [30]. By contrast, a German study revealed an association between higher CRP levels and more severe AKI, albeit the overall CRP levels in this study were somewhat lower than in the Finnish study [30,77].

Pentraxin-3 (PTX-3) belongs to the same pentraxin family as CRP. It is an acute-phase protein generated in various cells and tissues during the inflammatory stage

[10]. During acute PUUV infection, plasma PTX-3 levels are elevated, and the higher levels are associated with more severe AKI, as well as other variables reflecting disease severity [28]. PTX-3 is not used in clinical practice, at least not in Finland.

Procalcitonin (PCT) is a biomarker increasingly used in the detection of systemic bacterial infection. The level of PCT increases especially in bacterial infections and this has been used to differentiate bacterial infection from viral infections or other inflammatory states [10]. A German study showed that PCT elevation was typical in patients with acute PUUV infection, but it did not correlate with the severity of AKI nor the level of thrombocytopenia [78].

Several other biomarkers can predict the severity of PUUV infection. Serum indoleamine 2,3-dioxygenase (IDO) is elevated during the acute phase of the infection and correlates with increased disease severity [31]. Circulating plasma cell-free DNA (cf-DNA) is also elevated, but it correlates only with thrombocytopenia, leukocytosis, and the length of hospital stay, not with the severity of AKI [29]. Of adipokines, plasma resistin has been shown to be elevated in PUUV infection, and high resistin levels correlate with the severity of AKI and the overall severity of the infection [79]. In addition, levels of plasma YKL-40, also known as chitinase 3-like protein 1, are elevated during the acute phase of PUUV infection and are associated with the severity of AKI but not with thrombocytopenia [80]. Urinary biomarkers which can predict the severity of AKI are Type 2 cytokine transcription factor and neutrophil gelatinase-associated lipocalin [81,82]. Soluble urokinase-type plasminogen activator receptor (SuPAR) has not been studied earlier in hantavirus diseases. SuPAR is discussed in detail in Section 2.4.

2.2.4 Diagnosis

Diagnosis is based on clinical suspicion and serological confirmation. In the late 1970s and 1980s, diagnosis was based on the detection of a ≥ 4-fold increase in serum IgG antibodies [83]. Later, in 1996, the PUUV-IgM enzyme immunoassay was taken into routine use in Finland [84]. Because of the relatively long incubation period, almost all patients have IgM and IgG antibodies to the N protein at the onset of symptoms. However, 2–4% of patients do not present seroconversion until five days after the onset of symptoms [85]. A rapid immunochromatographic point-of-care IgM test is nowadays available [83]. It is possible to detect hantaviral RNA by polymerase chain reaction, using samples of peripheral blood, cerebrospinal fluid, and urine [83]. However, this method is not used routinely in clinical settings.

2.2.5 Treatment and prevention

There is no specific treatment available for PUUV infection so far. Treatment is supportive, aiming to maintain optimal fluid and electrolyte balance. Monitoring the patient is crucial in more severe cases during the hospital stay. Circulatory volume should be maintained, simultaneously avoiding fluid overload, which can lead to pulmonary edema. The most severe cases need intensive care and vasoactive support. Transient dialysis treatment is needed in 4–6% of patients [6–8,65]. Most PUUV infection cases are mild and remain without a confirmed diagnosis because the patient suffers a mild febrile illness at home without seeking medical assessment.

Antiviral treatment with Ribavirin has been studied, but the results have been conflicting [86–88]. There are two reports from Finland of the successful use of bradykinin receptor antagonist, icatibant, in severe cases of PUUV infection resistant to conventional treatment [89,90]. The outcome was favorable in both cases, but no larger studies have been conducted. In preventing hantavirus infections, no approved vaccines are available in the USA or Europe, while vaccines against HTNV and SEOV are in general use in China and the Republic of Korea [91]. The vaccines used are formalin-inactivated, and at least three doses are needed to provide protection [91]. However, a Korean study among army hospital patients did not show a significant difference in preventing severe AKI in vaccinated versus non-vaccinated patients [92].

The most important risk factors of PUUV infection are living in a building with holes allowing rodents to enter, living in rural areas, and cigarette smoking [3,21,49]. Increased awareness in endemic regions, appropriate protection from rodent excreta, and smoking cessation, are ways to reduce the risk of infection. Lifelong immunity is follows PUUV infection [93].

2.2.6 Long-term consequences

The overall prognosis of PUUV infection is favorable, and most patients achieve full recovery. Kidney function often fully recovers, but in some patients, greater proteinuria and hyperfiltration are seen 3–7 years after the infection [94]. In addition, systolic blood pressure has been found to remain higher in patients versus healthy controls [94]. At a control visit ten years after acute illness, these patients did not, however, present more hyperfiltration or proteinuria than the controls, and the difference in blood pressure was no longer significant [95]. PUUV-induced AKI leads to CKD very rarely. A German follow-up study revealed two out of 456

patients with creatinine levels higher than the upper limit of the reference value at a median of 17 months of follow-up [5]. One of these patients had glomerulonephritis diagnosed three months after acute PUUV infection and the other already had hypertension and coronary disease before the onset of PUUV infection [5].

Hormonal changes occur during acute PUUV infection, and long-term defects have also been reported [96]. In a Finnish follow-up study of 54 patients, 17% of them suffered from a hormonal deficiency in a 5-year follow-up period [96]. One patient was reported to have developed autoimmune polyendocrinopathy and hypophysitis [56]. A large registry-data study in Sweden showed a markedly increased risk of acute myocardial infarction and stroke within a week after acute infection [97]. Later, these patients also showed an increased risk of deep-vein thrombosis (IRR 45.9, 95% Cl 18–117.1) and pulmonary embolism (IRR 76.8, 95% Cl 37.1–159) 2–4 weeks after acute infection [98].

Recently, work from Sweden revealed that recovery from acute PUUV infection is sometimes slow, and the time to full recovery was more than three months in 47% and more than six months in 32% of the patients [99]. PUUV-infected patients reported significantly higher scores on the fatigue severity scale than the age, -gender- and a municipality-matched control group from the general population [99]. This is the only study concerning hantavirus infections and prolonged fatigue, though data on fatigue associated with other viral diseases, such as Ebstein-Barr virus infection, Dengue fever and Ross River virus have been reported [100–102]. Recently, several publications have come out showing COVID-19-related post-infectious fatigue syndrome [103].

In a large registry data-based Swedish study, an increased risk of 73% of developing lymphoma after PUUV infection was found [104]. A more recent study from Korea revealed an elevated risk of hematologic malignancies and solid-organ malignancies, especially leukemia and non-Hodgkin lymphoma, after HFRS, compared with the general population [105].

2.3 Pathogenesis and immunology of hantavirus disease

2.3.1 Increased capillary permeability

Increased capillary permeability is at the center of the pathogenesis of hantaviral infection. Plasma leakage into tissues is thought to be behind the various symptoms

and features of the infection. Integrins are transmembrane proteins acting as receptors for cell adhesion to the extracellular matrix as well as in cell-cell adhesion [106]. Integrins are the primary receptors for hantavirus to enter the cell [107]. Pathogenic hantaviruses use β3-integrin to enter the cell, whereas apathogenic hantaviruses seem to use β1-integrin [37]. β3-integrins are engaged in regulation of vascular integrity, endothelial permeability, and hemostasis [107]. They regulate vascular permeability through the effects of vascular endothelial growth factor (VEGF) [108]. β3-integrins and VEGF receptor 2 form functional complexes interacting with each other [109]. These complexes play a vital role in regulating endothelial barrier function [109]. Pathogenic hantaviruses inhibit the function of β3-integrin, thus leading to dysregulation of cellular functions in hantavirus disease [108,109]. Levels of VEGF are elevated in both hantaviral diseases HFRS and HCPS [108,110]. Nevertheless, a Slovenian study carried out to evaluate daily alterations of plasma and urinary VEGF concentrations showed no association between VEGF levels and disease severity in DOBV- and PUUV-infected patients [111].

2.3.2 Immune responses

Cytokines are soluble proteins acting as messengers of information between cells. They are produced by monocytes, macrophages, and lymphocytes in response to proinflammatory signals. Cytokines can be functionally divided into pro-inflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor (TNF)-α, and anti-inflammatory cytokines, such as IL-10, IL-1 receptor antagonist, and transforming growth factor (TGF)-β. Pro-inflammatory cytokines, especially IL-6 and TNF-α, are associated with fever and septic shock, and TNF-α can increase vascular permeability [112,113]. Cytokine levels are elevated in plasma, urine, and the kidneys of PUUV-infected patients [30,63,114–119]. In a Finnish study of 118 patients, high levels of plasma IL-6 were associated with several severity markers in PUUV-infected patients [30]. In earlier studies, plasma and urinary IL-6 levels have also been demonstrated to be elevated, but these levels did not correlate with each other, indicating local production of IL-6 in the kidneys [119]. TNF-α is another cytokine showing elevated levels at the acute phases of both HFRS and HCPS [120]. TNF-α is an inducer of nitric oxide (NO) synthase, which plays a role in capillary permeability and therefore may contribute to the pathogenesis of hantaviral disease. Impaired balance in the production of pro-inflammatory and regulatory cytokines has been proposed to be part of the pathogenesis of hantavirus disease. This

hypothesis is supported by the results of a Brazilian study of 21 HCPS patients [121]. The investigators found elevated levels of IL-6, TNF-α and interferon-γ, but decreased levels of regulatory TGF-β1 [121]. TGF-β1 levels were found to increase in the late phase of the disease compared with the early phase in German PUUV-infected patients [120].

Monocytes, macrophages, and dendritic cells activate CD8+ T lymphocytes, also known as cytotoxic T lymphocytes, by presenting the viral antigen to lymphocytes. PUUV nucleocapsid protein (N-protein) has been suggested to be the most significant immunomodulant antigen in activating CD8+ cells [122]. In hantavirus infection, increased amounts of circulating CD8+ cells have been detected in the blood and the kidneys in HFRS and the lungs in fatal HPCS [63,122,123]. Natural killer cells (NK cells) are a subpopulation of lymphocytes acting in innate immune responses to viral infections. Mature NK cells are traditionally identified as $CD3^-$ $CD56^+$ lymphocytes [124]. A Swedish study showed that NK cells are at low levels soon after the onset of symptoms but expand rapidly and remain upregulated for at least 60 days after acute infection [125]. NK cells may, though, have memory-like features. CD8+ memory cells are developed during the convalescence phase of PUUV infection, and these memory cells can be detected several years after infection [126,127].

The role of neutrophils in viral infection is uncertain. In hantaviral diseases, leukocytosis is often seen, and it is associated with the severity of AKI [82]. In humans, 50–70% of circulating leukocytes are neutrophils, and during acute PUUV infection, leukocyte counts shift to the left [9]. A major chemotactic factor for neutrophils is IL-8, which is upregulated in acute PUUV infection [63,128]. IL-8, along with neutrophil activation products, has been localized mainly in the tubulointerstitial space in the kidneys, suggesting neutrophil infiltration to the kidneys through the capillary endothelium [128]. High urinary IL-8 levels have been reported [82], indicating local production of IL-8 by the kidney epithelial cells in acute PUUV infection. Neutrophils have three main antimicrobial functions in the innate immune response. Firstly, phagocytosis. Secondly, degranulation, when they release an antimicrobial agent, and thirdly, the release of neutrophil extracellular traps (NETs). The last one is a form of apoptosis, and it is called NETosis [129]. NETs are net-like structures of double-stranded DNA coated with histones and antimicrobial molecules [130]. Hantaviruses have been shown to stimulate neutrophils to release NETs, and NETs have also been detected in kidney biopsy samples by immunofluorescence analysis [130].

The complement system takes part in the innate and adaptive immune systems. The complement system can be activated through three different pathways: the classical route, the alternative route, and the lectin route. All routes are activated differently, but they all converge on component C3, an essential functional component of the complement system. The end-product of the complement cascade is the cytolytic membrane attack complex (SC5b-9). When the complex is formed without a target cell membrane, C5b-9 binds to S-protein or clusterin, forming soluble SC5b-9 complexes [131]. Two Finnish studies have concerned the role of the complement system in PUUV infection [132,133]. In the first study, involving 25 patients, complement activation was present in 23 (92%) patients, and the classical pathway of activation was associated with a more severe clinical course of infection [132]. The second study was more extensive, with 61 patients, and it revealed complement activation mainly via an alternative route [133]. SC5b-9 was significantly elevated at the acute stage, whereas levels of C3 were decreased, indicating activation of the alternative pathway. Higher SC5b-9 levels were found in the patients with pulmonary abnormalities, in addition to lower levels of C3 as a result of enhanced consumption [133]. In a recent study of four fatal PUUV infection cases, severe pathology was found in the lungs, spleen, liver, and hypophysis but not in the kidneys. Deposits of C3d and SC5b-9 were found particularly in the lungs at autopsy. Premortem complement activation was also seen [134].

SC5b-9 can increase endothelial permeability by ligating $\alpha_v\beta_3$-integrin of the lung endothelium in rats [135]. SC5b-9 also promotes increased permeability in human endothelial cells by releasing bradykinin and platelet-activating factor [136]. Bradykinin is a multipotent polypeptide formed by the kallikrein-kinin cascade. It promotes inflammatory mediator and NO release and increases vascular permeability and vasodilatation [137]. In an experimental model of hantavirus infection, a dramatic increase in permeability of endothelial cells was seen after activation of the kallikrein-kinin system and liberation of bradykinin [138]. In line with this observation, there are two reports of seriously ill PUUV-infected patients, resistant to supportive therapy, treated successfully with icatibant, a bradykinin antagonist drug [89,90].

2.3.3 Host genetics

Host-related factors influence the clinical outcome of hantavirus disease. Human leukocyte antigens (HLAs) are cell-surface antigens presenting pathogen-derived

antigens to the T cells, initiating adaptive immune responses. In a Finnish study of 74 patients, HLA alleles B8, C4A*Q0, and DRB1*0301 were associated with more severe disease [139]. HLA B8 was found in all the patients presenting with clinical shock on admission and in 9 of the 13 (69%) patients who needed transient dialysis treatment [139]. The same haplotype HLA B8 DRB1*0301 leads to failure to respond to the hepatitis B vaccine [140], and it is also associated with a more rapid development of AIDS in human immunodeficiency virus (HIV)-infected patients [141]. In contrast, haplotype HLA-B27 contributes to a more favorable course of the disease in HIV infection, and therefore, a study to evaluate PUUV-infected patients has also been conducted [142]. HLA-B27 was associated with a milder course in PUUV infection, and this haplotype was under-represented in the study population as compared with the general population [142].

2.4 Soluble urokinase Plasminogen Activator Receptor (SuPAR) as a biomarker in infectious diseases

Urokinase-type plasminogen activator receptor (uPAR) is a multifunctional glycoprotein with a molecular weight of 55–60 kDa [143]. The expression of uPAR is increased during infectious and inflammatory states [32,144]. uPAR is expressed by various immunologically active cells, such as monocytes, activated T-lymphocytes, macrophages, neutrophils, endothelial cells, and kidney podocytes [32,144]. SuPAR, the soluble form of uPAR, is formed when G-protein (GPI)-anchor-bound uPAR is cleaved and released from the cell surface [32]. SuPAR can be detected in various body fluids such as plasma, urine, and cerebrospinal fluid [32]. The level of plasma suPAR is associated with disease severity in various infectious diseases and has been proposed to be a prognostic tool in evaluating patients. Plasma SuPAR levels are associated with mortality in cases of malaria [145], tuberculosis [146], and HIV -infection [147,148]. In bacteremic patients, plasma suPAR has been shown to be associated with poor outcome and to predict mortality [149–151]. In critically ill patients, suPAR can serve as a prognostic tool on admission to the intensive care unit, and lower levels are positive predictors of overall survival [152]. In a Finnish study by Uusitalo-Seppälä et al., high suPAR levels predicted case fatality and severe sepsis in patients with suspected infection in an emergency room [153]. Levels of cerebrospinal fluid suPAR are higher in patients with purulent meningitis than in those without proven meningitis or those with lymphocytic meningitis [154]. A retrospective study of one hundred patients with Crimean-Congo hemorrhagic

fever (CCHF) showed higher levels of plasma suPAR in patients with CCHF than controls, and suPAR levels predicted mortality [155]. There are no previous studies on suPAR levels in hantavirus diseases.

2.5 Radiological findings in Puumala virus infection

2.5.1 Chest X-ray, computed tomography and renal ultrasound findings

Pathological chest X-ray findings are common in acute PUUV infection. Abnormalities have been demonstrated in 10–35% of PUUV-infected patients [5–7,156–158]. The most common finding is pleural effusion followed by atelectasis, opacities, venous stasis, and pulmonary edema [16]. Cardiac enlargement has also been found during acute PUUV infection [7,157,158]. Pathological X-ray findings have been shown to correlate with disease-severity markers, i.e., length of hospital stay, change in body weight, and high serum creatinine concentrations [156,158]. The incidence of abnormal pulmonary findings during the acute PUUV infection are demonstrated in Table 2.

Table 2. Abnormal thoracic cavity findings in acute PUUV infection.

Author	Country	Imaging modality	Number and percentages of abnormal findings	Most frequent findings	Reference
Settergren et al. 1989	Sweden	Chest X-ray	1/10 (10%)	Pleural effusion	[6]
Mustonen et al. 1994	Finland	Chest X-ray	15/94 (16%)	Pleural effusion, parenchymal infiltration, pulmonary edema, cardiac enlargement (2 patients)	[7]
Kanerva et al. 1996	Finland	Chest X-ray	27/97 (28%)	Pleural effusion, parenchymal infiltration, atelectasis, pulmonary edema	[156]
Paakkala et al. 2004	Finland	Chest X-ray	121/344 (35%)	Pleural effusion, atelectasis, pulmonary infiltration, cardiac enlargement (30 patients)	[158]
Mäkelä et al. 2009	Finland	Chest X-ray	19/62 (31%)	Pleural effusion, atelectasis, pulmonary infiltration, cardiac enlargement (3 patients)	[157]
Latus et al. 2015	Germany	Chest X-ray	49/214 (23%)	not reported	[5]
Linderholm et al. 1992	Sweden	Lung CT	10/19 (53%)	Interstitial or alveolar infiltrates, pleural effusion	[159]
Paakkala et al. 2012	Finland	Chest HRCT	12/13 (92%)	Atelectasis, pleural effusion, intralobular and interlobular septal thickening	[160]
Rasmuson et al. 2013	Sweden	Chest HRCT	14/27 (52%)	Pleural effusion, pulmonary oedema, enlarged thoracic lymph nodes, pericardial effusion, and pneumonic infiltrate	[161]

Abbreviations: X-ray=radiograph, CT=computed tomography, HRCT=high-resolution computed tomography.

Of studies concerning CT and HRCT imaging, two of them were carried out to evaluate the association between pulmonary and clinical findings. Neither of these Swedish studies showed differences in serum creatinine levels between the patients with and without abnormal CT or HRCT findings [159,161].

Ultrasonographic (US) examination is a cheap and widely used method to examine intra-abdominal organs, including the kidneys. In a Finnish renal US study of 20 patients, renal length was increased in all patients at the acute phase of PUUV infection as compared with the control stage [162]. Fluid collections were found in 13/20 of the patients, and the severity of US findings was associated with fluid volume overload [162]. Only qualitative evaluation of fluid collections in the perirenal, pleural, and pericardial spaces, and ascites was made, and the associations between fluid collections and change in body weight and maximum daily urine excretion were the only significant ones [162].

Recently, a study of thirty PUUV-infected patients investigated by means of abdominal CT scanning was published [17]. Perirenal or retroperitoneal fat stranding

and/or perirenal fascial thickening was found in most patients with acute PUUV infection, but the association between these findings and the clinical severity of the infection was not studied [17].

2.5.2 Magnetic resonance imaging findings

Renal magnetic resonance imaging (MRI) has been studied as well. In a Finnish study of 20 patients, renal length, parenchymal volume, and thickness were significantly greater during the acute stage compared with measures at the control visit [163]. There were no significant associations between renal MRI findings and clinical or laboratory findings [163]. MRI was assessed as being superior to US in showing mild edema and fluid collections [163].

The size of the spleen was also studied by means of MRI in the same 20 patients. Enlargement of the spleen was found in every patient during acute PUUV infection, but no associations with disease severity were found [164]. Neither was there a correlation between the changes in length of the spleen and the kidneys [164]. There are no other previous reports on abdominal MRI findings in PUUV infection.

MRI examination has also been used to explore the brain and, specifically, pituitary gland involvement in PUUV infection. Pituitary gland involvement may occur during acute PUUV infection, presenting as edema and hemorrhages [16]. In a study of 45 patients examined by means of brain MRI, pituitary abnormalities were found in six patients, of which two had acute hemorrhages [165]. Some cases of hypophyseal hemorrhages and panhypopituitarism after hantaviral infection have been described [166]. Some of the patients had transient loss of vision and they also suffered from headache [54,61,62,167]. A few cases have been reported with cytotoxic lesion of the corpus callosum, and acute disseminated encephalomyelitis during acute PUUV infection [168–172].

Cardiac findings are common in acute PUUV infection [157,161]. German colleagues have presented a case report of PUUV-induced acute myocarditis diagnosed by means of MRI [173]. They concluded that multiparametric cardiac magnetic resonance would serve to detect and follow-up patients with hantavirus-induced myocarditis.

2.6 Smoking

2.6.1 Structural and functional changes in the airways

Smoking results in many different changes in the airways. Cigarette smoke (CS) includes more than 4,500 substances, including tar, acrolein, acetaldehyde, formaldehyde, free radicals, and nitric oxide. The mucociliary system is the first defense line against pathogens in the lungs. Ciliated epithelial cells and goblet cells form most of the luminal surface of the large airways and they are joined together by tight junctions [174]. Histopathological findings in the airways of a cigarette smoker include a reduction of cilia in the epithelium and an increase in the number of goblet cells, hypertrophy of submucosal glands, and squamous cell metaplasia [175].

Furthermore, the movement of cilia is decreased [175]. In addition, immunological changes have been observed. In studies of bronchoalveolar lavage fluid, increased numbers of CD8+ lymphocytes have been found in smokers, whereas the amount of CD4+ cells was decreased, resulting in a lower CD4+/CD8+ ratio [18]. Immunoglobulin G levels have been observed to be elevated in the bronchial fluid of current smokers versus nonsmokers [18].

Alveolar macrophages (AMs) perform in a normal lung as key phagocytic cells. Cigarette smoke influences alveolar macrophage recruitment, phenotype, immune functions, and homeostasis [176]. Firstly, CS causes oxidative stress, producing chronic low-grade inflammation, and inflammatory cells including macrophages are recruited into the lung [176]. Secondly, CS changes the phenotype of alveolar macrophages towards immature, undifferentiated monocyte-like macrophages, reducing their effectiveness [176]. Thirdly, CS alters the immune functions of AMs. Cigarette smoking leads to impaired phagocytosis, imbalance in proteinase/anti-proteinase release from AMs, and dysregulation of production of reactive oxygen species (ROS). Lastly, CS increases the amount of extracellular iron from AMs, resulting in oxidative damage [176]. NK cells are also affected by CS. There are results showing both stimulation and suppression of NK cell activity, but in the airways, activation of NK cells has been found to be independent of smoking status [177].

2.6.2 Smoking and infection

The mechanism of how smoking increases the risk of infection is multifactorial. In addition to structural and functional changes in the airways, smoking affects both cell-mediated and humoral immune responses. Smokers have an approximately 30% higher peripheral blood leukocyte count than nonsmokers [18]. The underlying mechanism has been proposed to be accelerated bone marrow stimulation via alveolar macrophage-induced proinflammatory factor enhancement [18]. All cell types, including lymphocytes, are increased. Although information concerning lymphocyte distribution is somewhat conflicting, most studies indicate that the number of CD8+ lymphocytes, as well as their activation and function, is increased in heavy smokers [178]. In heavy smokers, the number of CD4+ cells has been shown to be decreased, thus leading to increased susceptibility to infections [18]. In the lung, cigarette smoke suppresses the early proinflammatory cytokines but enhances the production of late proinflammatory mediators, such as IL-6, IL-8, INF-γ, TNF-α, IL-1β and IL-18, thus contributing to prolonged inflammation and lung injury [179].

In smokers, NK cell activity in peripheral blood is decreased [18]. NK cells are vital in defense against a viral infection; therefore, smoking may lead to increased susceptibility to viral infection. Smoking also results in alterations in humoral immunity. Peripheral immunoglobulins are 10–20% lower in smokers as compared with nonsmokers, but this is a reversible alteration and is normalized after smoking cessation [18,180].

Smoking is an independent risk factor of community-acquired pneumonia. In a large case-control study carried out in Spain, current smokers and ex-smokers had an increased risk of community-acquired pneumonia [181]. Several studies have also shown smokers' increased risk of invasive pneumococcal pneumonia compared with nonsmokers [182–184]. In addition, smoking results in an increased risk of tuberculosis and Legionella pneumonia [185,186]. A recent meta-analysis also revealed an increased risk of invasive meningococcal disease in smokers [187].

The outcome of different infections in smokers has been reviewed by Huttunen and coworkers [19]. Overall, the data indicated that the rates of pneumonia-associated mortality and tuberculosis-associated mortality are increased among smokers [19]. Furthermore, smoking is associated with increased risks and worsened outcomes of several other bacterial infections, such as Clostridioides difficile infection, Helicobacter pylori infection, and anal abscess and fistula [188]. Smoking increases the risk of periodontal infection and results in a more severe outcome

[19,188]. In addition, postoperative wound-healing complications are more frequent in smokers than in nonsmokers [189].

Viral infections have also been studied from the perspective of the effects of smoking. Smoking may predispose individuals to more severe influenza, and it increases the overall risk of contracting influenza [19,190]. The risk of a common cold is increased among healthy smokers compared with nonsmokers [191]. Recently, preliminary lines of evidence suggest that smokers present with more severe COVID-19 infection [192]. Furthermore, cigarette smoking increases the risk of varicella pneumonitis and human papillomavirus infections [18]. Passive smoking and exposure to environmental cigarette smoke have been shown to predispose children to pneumococcal and meningococcal disease, otitis media, bronchitis, bronchiolitis, and pneumonia [19].

Two previous case-control studies carried out in Belgium and Finland have shown cigarette smoking to be an independent risk factor of PUUV infection, with ORs of 9.1 and 3.1, respectively [20,21]. No previous studies exist concerning the association between smoking and the severity of hantaviral diseases.

2.6.3 Smoking and the kidneys

Smoking increases the risk of developing chronic kidney disease (CKD) in the general population [193,194]. The role of cigarette smoking in the development of AKI has been less well studied. Smoking activates the renin-angiotensin system, resulting in a rise in blood pressure. Both cardiac output and peripheral resistance increase as a result of cigarette smoking, the principal causative agent being nicotine [195]. A study of healthy volunteers showed a rise in blood pressure and a decrease in glomerular filtration rate and renal blood flow after nicotine exposure [196]. These hemodynamic effects may contribute to CKD progression. The progression of CKD is shown in smokers with diabetic nephropathy, IgA nephropathy, and idiopathic membranous nephropathy [194]. In addition to hemodynamic alterations, there are local changes due to smoking. Smoking-induced oxidative stress leads to endothelial dysfunction and elevates the production of ROS in various organs, including the kidneys. Potential pathophysiologic mechanisms leading to smoking-induced renal injury include tubulotoxicity, toxic effects on endothelial cells, an increase in renal vascular resistance, arteriosclerosis of the renal and intrarenal arteries, increased sympathetic nerve activity, and increases in blood pressure and heart rate [197].

The effects of CS on the development of AKI are far less well studied. Cigarette smoke is known to affect mitochondria. Arany and coworkers have demonstrated a mouse model in which chronic nicotine exposure results in an increase in protein p66shc expression. This results in mitochondrial ROS production and depolarization of mitochondria, leading to injury in the proximal tubule cells [198]. The mechanisms by which CS, and particularly nicotine, take part in the development of AKI may be somewhat consistent with mechanisms in the progression of chronic kidney injury. Nevertheless, there may also be different mechanisms which still remain to be elucidated.

2.7 Alcohol consumption and markers of alcohol use

2.7.1 Effect of alcohol on host defense

Both chronic and acute alcohol consumption affect the immune system and the ability of the body to defend itself against pathogens. Chronic alcohol abuse induces alterations in the immune system and therefore predisposes individuals to viral and bacterial infections [26]. Acute and chronic alcohol exposure have opposite effects on inflammatory cell activation. Acute alcohol exposure suppresses cytokine and chemokine responses, whereas chronic exposure enhances the production of proinflammatory cytokines, especially that of TNF-α [199]. Alcohol also affects neutrophils. It increases ROS production and decreases the phagocytic capacity of neutrophils and macrophages [26]. In addition, alcohol affects the adaptive immune system. Chronic alcohol use leads to a decrease in the total amount of lymphocytes, and it also shifts homeostasis towards the memory T-cell phenotype [200]. Chronic alcohol abuse also influences B cells. Circulating B cell numbers decrease as a result of alcohol intake. In particular, the number of B-2 B cells (conventional B cells), which are responsible for forming an immunological memory and producing antibodies, is reduced [200]. Although the number of B cells is reduced, the use of alcohol results in higher levels of circulating immunoglobulins [200]. Patients with alcoholic liver disease have higher blood IgG and IgA levels than patients without the disease [201]. Also, IgA and IgM levels in patients with heavy alcohol consumption have been shown to be elevated [202]. IgE levels are elevated among patients with chronic alcohol consumption [200].

Alcohol abuse may predispose people to various infectious diseases. Several lines of evidence show increased susceptibility to pulmonary infections such as pneumonia caused by Streptococcus pneumoniae, Klebsiella pneumoniae, Hemophilus influenzae, and Legionella pneumophila [25]. A relative risk of 1.83 (95% CI: 1.3–2.57) as regards community-acquired pneumonia in patients who used alcohol was found in a meta-analysis of 14 studies [203]. Furthermore, a systematic review showed a relative risk of 2.94 (95% CI:1.89–4.59) as regards tuberculosis in patients with heavy alcohol use or alcohol use disorder [204]. The same review included several studies indicating a more severe course of tuberculosis and higher relapse rates [204]. In animal models, alcohol exposure has been shown to be a predisposing factor as regards viral pulmonal infections, such as respiratory syncytial virus pneumonia [205]. Furthermore, chronic alcohol exposure worsens the clinical outcome of various infections. Of systemic bacterial infections, Listeria monocytes and Salmonella infections may manifest with worsened clinical outcome among alcoholics [25]. In an experimental mouse model of Klebsiella pneumoniae infection, alcohol-fed mice had bacteremia 37 times more often, and they had higher mortality as compared with controls [206]. The influence of alcohol exposure on hantavirus diseases has not been established.

2.7.2 Markers of alcohol consumption

Gamma-glutamyl transferase (GGT) is a membrane-bound glycoprotein enzyme found in the kidneys, liver, pancreas, and brain [207]. In the liver and biliary tract, GGT is mainly found in the hepatocytes and biliary epithelial cells [208]. Since GGT is responsible for the extracellular metabolism of glutathione, elevated GGT levels may also indicate hepatic oxidative stress induced by alcohol consumption or an overweight condition [209]. GGT activities have also been linked to cardiovascular diseases, diabetes, and metabolic syndrome, and in addition seem to predict cardiovascular and all-cause mortality [209]. GGT has a 70–73% sensitivity to detect alcohol heavy consumption [210]. In addition to alcohol use, GGT levels may rise in various other conditions, such as obesity, smoking, drug use, and some infections, especially in hospital settings [211]. All these conditions increase oxidative stress. Due to the many confounding factors, the specificity of GGT in detecting heavy alcohol consumption is relatively low. In a non-selected study population of 1863 patients, the specificity among men as regards the detection of heavy alcohol

consumption was 74% [212]. GGT levels normalize two to three weeks after the cessation of alcohol use if the elevation is due to alcohol in the first place [211].

Carbohydrate-deficient transferrin (CDT) is another biomarker widely used to detect alcohol consumption. Transferrin is a protein synthesized and secreted by the liver. The primary function of transferrin is to transport iron. Transferrin has glucan chains with a varying number of terminal sialic acid residues bound to these chains [211]. In human serum, the most abundant glycoform is tetrasialotransferrin, accounting for over 80% of the total transferrin [213]. Excessive alcohol intake interferes with the synthesis of transferrin by decreasing sialylation in the hepatocytes [214]. In the blood, this results in increased disialo- and asialotransferrin, leading to CDT. The proportion of carbohydrate-deficient transferrin to total transferrin (%CDT) is a useful measure nowadays most commonly used in Europe [214]. Standardization of reference limits has been complicated because different methods cover variable fractions of different glycoforms, and therefore different method-based reference limits have been used [213]. Levels of %CDT increase when alcohol consumption exceeds 50–80g/day for two to three weeks [211]. In a Finnish study, with a cut-off limit of 2.6%, the specificity as regards heavy alcohol intake was 98%, but the sensitivity was only 63% [27]. A reference value of 2.5% is commonly used in Finnish laboratories to detect heavy alcohol intake. The mean normalization time of %CDT is 16 days [27]. %CDT has also been shown to be a reliable biomarker for detecting heavy alcohol consumption in patients with liver damage [214]. A physiological progressive increase of CDT occurs during pregnancy in the absence of alcohol use [215]. Other confounding factors may be anorexia, smoking and acute trauma with blood loss [211].

GGT-CDT combination marker has been established to improve specificity without losing sensitivity in detecting alcohol consumption. The mathematical value of the combination marker GGT-CDT is calculated by using the equation $0.8 \times \ln(\text{GGT}) + 1.3 \times \ln(\%\text{CDT})$ [210]. GGT-CDT reacts when alcohol consumption exceeds 40g per day [216]. In detecting heavy alcohol drinking, the sensitivity of the combination marker was 90% and the specificity 98% when cut-off limits of 4.1 for men and 3.8 for women were used [27]. Later, cut-off values of 4.3 for men and 3.8 for women were proposed [217]. In addition, the level of GGT-CDT has been found to correlate better with the amount of self-reported alcohol use than GGT or CDT alone [27]. It can also be used in the follow-up of abstinence, since it declines after alcohol cessation, with a mean normalization rate of 2–3 weeks [27].

Ethyl glucuronide (EtG) is an ethanol (EtOH) metabolite with a molecular weight of 222 g/mol [218]. It is formed in the liver by enzymatic conjugation of ethanol

with glucuronic acid. Another metabolite is ethyl sulfate, formed from ethanol by sulfotransferase [219]. These metabolites can be analyzed by immunological or liquid chromatography-mass spectrometry techniques [216]. They can be detected in serum, whole blood, urine, and hair [216]. In urine, EtG can be measured for up to 90 hours [219], and urine is the most common sample type used. Urinary EtG can also be detected after nonintentional exposure to alcohol, such as in non-alcoholic beer, pharmaceutic products, alcohol-containing hand disinfection, and even after consuming yeast and sugar together [218]. Therefore, a cut-off limit of 500 ng/ml is used to avoid false positive results [220]. False negative urinary EtG results may result from acute urinary tract infection due to Escherichia coli, when β-glucuronidase breaks down EtG but does not break down ethyl sulfate [221]. The detection time in urine is dose-dependent, and an approximate dose of 0.5 g alcohol/kg can be detected for up to 48 hours [220].

Phosphatidylethanol (PEth) is an abnormal phospholipid formed from phosphatidylcholine only in the presence of ethanol. Without ethanol, phosphatidic acid is formed instead of PEth [222]. Therefore, the specificity of PEth to indicate alcohol consumption is high, and there are no false-positive results [223]. In addition, the sensitivity of PEth is very high, reaching 99% [224]. PEth is enriched in the erythrocytes in the blood after regular ethanol intake of more than one week [222]. The half-life of PEth in the circulation in alcoholics is approximately four days [224] and PEth can be detected in blood samples for four weeks after the cessation of alcohol intake [222]. A single dose of ethanol does not elevate PEth to measurable concentrations. PEth also shows a better correlation with the reported alcohol intake as compared with traditional markers of alcohol abuse [224]. PEth is measured in the fresh whole blood samples by means of liquid chromatography tandem mass spectrometry [222].

3 AIMS OF THE STUDY

The present study was aimed at investigating factors associated with the clinical severity of PUUV infection. The specific aims were:

1. To assess whether cigarette smoking affects the clinical course of PUUV infection and to evaluate the prevalence of cigarette smoking in PUUV-infected patients (I).

2. To evaluate whether alcohol consumption affects the clinical outcome of PUUV infection and to assess changes in liver enzyme levels during acute PUUV infection (II).

3. To study pancreatic involvement in acute PUUV infection and its association with patients' symptoms (II).

4. To evaluate the amount and distribution of abdominal fluid below the diaphragm and the amount of pleural fluid by means of magnetic resonance imaging, and the possible relationship between fluid collections and the patient's clinical symptoms and findings during acute PUUV infection (III).

5. To evaluate the association of plasma suPAR levels with the severity of PUUV infection (IV).

4 PATIENTS AND METHODS

4.1 Patients (Studies I–IV)

The study was conducted at Tampere University Hospital and Tampere University, Finland. Patients with serologically confirmed PUUV infection were treated between 1982 and 2014. Altogether, 547 patients were included in Studies I–IV. As presented in Table 3, all studies partly included the same patients. Of the patients in Study III, 23/27 were also included in Study II.

Table 3. Patients presented in different time periods according to the hospital treatment time.

Hospital treatment time periods	1982-1999	2000-2004	2005-2007	2008-2014	Total number of patients
Study I	257	43	31	26	357
Study II			34	26	66
Study III			27		27
Study IV		42	40	15	97

Patients in all studies were relatively healthy. None of them had CKD before the onset of PUUV infection. Table 4 shows the similarity in the distribution of comorbid diseases.

Table 4. Comorbid diseases of the patients before the onset of acute PUUV infection.

Comorbidities	Study I (N=357) 1982-2012	Study II (N=66) 2005-2014	Study III (N=27) 2005-2007	Study IV (N=97) 2001-2009
Hypertension	24 (6.7%)	7 (10.6%)	3 (11.0%)	9 (9.3%)
Hypercholesterolemia	7 (2.0%)	2 (3.0%)	0 (0%)	3 (3.1%)
Coronary artery disease	8 (2.2%)	2 (3.0%)	0 (0%)	2 (2.1%)
Diabetes	7 (2.0%)	3 (4.6%)	1 (3.7%)	2 (2.1%)
Atrial fibrillation	4 (1.1%)	2 (3.0%)	1 (3.7%)	4 (4.1%)
Asthma	8 (2.2%)	2 (3.0%)	1 (3.7%)	4 (4.1%)
Hypothyroidism	6 (1.7%)	0 (0%)	0 (0%)	0 (0%)
Rheumatoid arthritis/spondyloarthropathy	3 (0.8%)	2 (3.0%)	2 (7.4%)	3 (3.1%)

4.2 Methods

4.2.1 Summary of Studies (Studies I–IV)

Table 5 summarizes the design, methods, and objectives of Studies I–IV.

Table 5. Summary of Studies I–IV.

	Study I	Study II	Study III	Study IV
Study period (years)	1982-2012	2005-2014	2005-2007	2001-2009
Number of patients	357	66	27	97
Design of the study	Retrospective cohort	Retrospective cohort	Prospective cohort	Prospective cohort
Method of data collection	Hospital medical records and posted questionnaire between January 2012 and March 2012	Hospital medical records and frozen blood samples	Hospital medical records and MRI analysis	Hospital medical records and frozen blood samples
Objective of the study	Evaluate the effect of cigarette smoking on the severity of PUUV-induced AKI and evaluate the prevalence of cigarette smoking in PUUV-infected patients	Evaluate whether alcohol drinking affects the clinical outcome of PUUV infection and assess changes in liver enzymes during acute PUUV infection. This study also aimed to evaluate whether acute pancreatitis is present during acute PUUV infection	Evaluate the amount and distribution of fluid in the intra- and retroperitoneal space and pleural space during acute PUUV infection and its relationship to symptoms and clinical findings	Evaluate the association between suPAR and the severity of acute PUUV infection

4.2.2 Diagnosis of Puumala virus infection (Studies I–IV)

PUUV infection was serologically confirmed in all patients. Diagnosis in the 1980's was made by detecting a fourfold increase in IgG levels [41]. In 1996 an "in-house" PUUV-IgM enzyme-linked immunosorbent assay (ELISA) was taken into routine use in the diagnosis of acute PUUV infection [84].

4.2.3 Laboratory and clinical variables (Studies I–IV)

During the hospital stay, basic laboratory tests were carried out by standard methods at the Laboratory Center of Pirkanmaa Hospital District, later named Fimlab Laboratories. Basic laboratory tests, e.g., serum or plasma creatinine and plasma CRP concentrations, blood leukocyte and platelet counts and hematocrit, were carried out according to the patient's clinical needs. The highest and lowest values during the

hospital stay were designated as maximum and minimum values. Measurements of the levels of alcohol markers (Study II) and plasma suPAR (Study IV) are discussed later in detail. In Study III, the urinary albumin excretion rate (UAER) was measured during the hospital stay at median of 0 days (range -4–1 days) with respect to MRI. In Study IV, blood samples for cf-DNA, IL-6, CRP, PTX3, plasma creatinine and kynurenine, tryptophan, and blood cell count were obtained for up to five consecutive days.

Clinical variables, such as blood pressure and weight, were measured daily during the hospital stay. The length of hospital stay reflects the overall severity of the illness, and it is counted in days. Furthermore, a change in body weight (kg) reflects fluid retention and balance between oliguric and polyuric stages. The patient's symptoms were recorded accurately on admission and daily during hospitalization.

Severe AKI was defined in all studies according to KDIGO clinical practice guidelines for AKI stage 3 as a plasma creatinine level equal to or more than 353.6 μmol/L [225].

In Study III, urinary trypsinogen (U-Trypsin) levels under the detection limit were presented as 1.56 ng/L, the lowest measurable value. Acute pancreatitis was defined as a serum amylase level threefold above the upper limit of normal and the presence of abdominal pain.

4.2.4 Smoking status (Study I)

Data on smoking history was collected via a questionnaire sent to 494 patients between January 2012 and March 2012. Of the original study population of 569 patients in Study I, 45 were deceased, two were living abroad, and for 28 patients addresses were not available and they were therefore excluded. Of 494 patients, 357 (72%) answered and comprised the final study cohort.

To evaluate the validity of the questionnaire, the answers of 114 respondents who were interviewed by the research nurse during the hospital stay were compared with the data obtained from the questionnaire. There were differences in only two (1.8%) out of 114 answers. Therefore, the questionnaire's validity and the respondents' memory should be considered reasonable.

The questionnaire contained six questions concerning smoking habits before and at the onset of PUUV infection. The duration of smoking and the daily number of cigarettes were asked about. Analysis of non-respondents was carried out to evaluate

the possible bias due to willingness to participate. The questionnaire is available in the appendix of this thesis.

According to the smoking history at the time of PUUV infection, the patients were divided into three groups: current smokers, ex-smokers, and never-smokers. The patients were further divided into current smokers and nonsmokers (including never-smokers and ex-smokers) to evaluate the effect of current smoking.

4.2.5 Alcohol markers and definition of alcohol use (Study II)

In Study II, alcohol markers were measured in thawed blood and urine samples collected during the hospital stay and stored at -70 °C. Control samples were obtained at a median of 24 (17–76) days after the onset of fever and were stored frozen at -70 °C until the analyses were performed. The concentrations of S-GGT, S-CDT, U-EtG, serum alanine aminotransferase (S-ALT), serum- and urinary (U) creatinine (S/U-creatinine), serum cystatin C (S-Cys C), serum- and urinary amylase (S/U-AMYL) and urinary trypsinogen (U-Trypsin) were measured by accredited methods at the Laboratory of Seinäjoki Central Hospital, Seinäjoki. To improve the sensitivity and specificity of detecting heavy alcohol consumption, the combination marker GGT-CDT was calculated by using the equation GGT-CDT = 0.8 × ln (GGT) + 1.3 × ln (CDT). Cut-off limits of 3.8 for women and 4.3 for men were used [217]. Regarding U-EtG, 500 ng/mL was set as a cut-off value to reflect recent alcohol consumption. The convalescence stage value of GGT-CDT was used to avoid the confounding effect of acute infection on GGT levels.

A patient was designated as an alcohol heavy user when the combination marker GGT-CDT exceeded the cut-off limit at the control stage. When U-EtG reached 500 ng/mL, but GGT-CDT remained under the cut-off limit, the patient was designated as a light drinker. When neither of the alcohol markers exceeded the cut-off limits, the patient was defined as an abstainer. In addition, patients were divided into two groups: alcohol drinkers (including heavy drinkers and light drinkers) and abstainers. Reference values of the variables used are presented in Table 6.

Table 6. Reference values used in Study II.

Laboratory value	Men	Women
S-Creatinine	60-100 µmol/L	50-90 µmol/L
S-Cystatin C	< 50 years 1.2 mg/L >50 years 1.4 mg/L	
S-ALT	<50 U/L	<35 U/L
S-GGT	<60 U/L	<40 U/L
S-CDT	<2.5%	
S-GGT-CDT	4.3	3.8
U-EtG	<500 ng/mL	
S-Amyl	25-120 U/L	
U-Trypsin	1.56-100 ng/mL	

4.2.6 Magnetic resonance imaging (Study III)

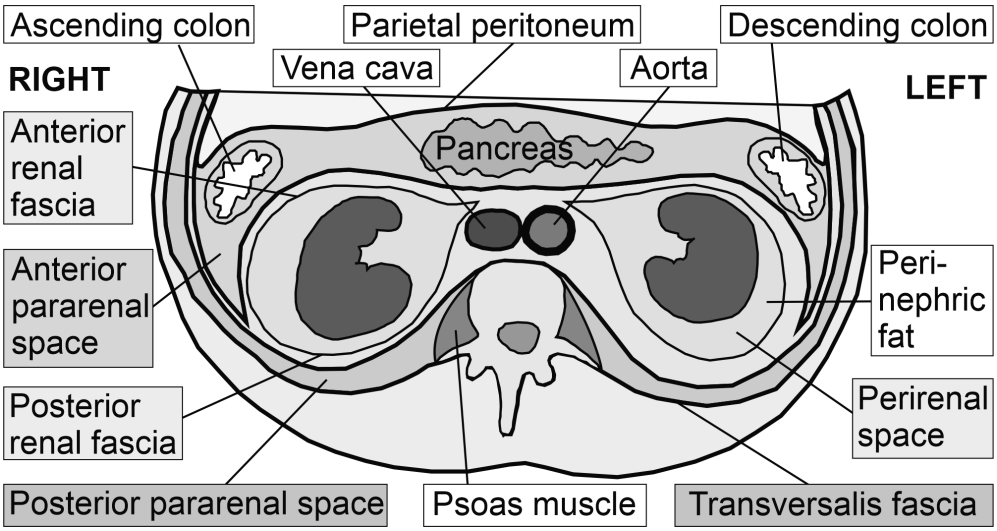
In Study III, abdominal MRI was performed during the hospital stay, at a median of six days (range 3–11) after the onset of fever. MRI was performed with a 1.5-T imager, Siemens Magnetom Avanto (Erlangen, Germany) using a combination of spine and body matrix coils. The MR images were assessed by an experienced radiologist (Tommi Kiekara), unaware of the patients' clinical data. All the measurements were carried out with a Sectra IDS7 workstation.

MRI was performed with the patient in a supine position and the abdomen was imaged in coronal and axial directions. The caudal parts of the pleura were visible in the images. The greatest thickness of the fluid was measured in millimeters (mm). Pleural fluid was assessed on both sides separately and the data then added together. Perirenal and pararenal fluid was evaluated on both sides and the data then added together.

The schematic cross-section of the abdominal region is shown in Figure 1. The perirenal space is located between the kidney and the anterior and posterior renal fascia. The pararenal space lies between the posterior renal fascia and the transversalis fascia. Perihepatic fluid is located next to the liver, perisplenic fluid next to the spleen and fluid is also located next to the psoas muscle. The Pouch of Douglas is the deepest cavity of the retroperitoneal space, and it is located posterior to the bladder. Fluid easily accumulates in the Pouch of Douglas without clinical relevance. Therefore, this fluid was excluded from the analyses. Intraperitoneal fluid

includes perihepatic and perisplenic fluid and fluid next to the psoas muscle. Retroperitoneal fluid includes perirenal and pararenal fluid.

Figure 1. Schematic cross-section of the abdominal region showing locations of anterior pararenal space, perirenal space and posterior pararenal space. (Adopted from Study III, Tervo et al. The presence of intraperitoneal, retroperitoneal and pleural fluid in acute Puumala hantavirus infection. Infectious Diseases (London) 2022; Dec 23;1-9).



4.2.7 Soluble urokinase-type Plasminogen Activator Receptor determination (Study IV)

In Study IV, EDTA-treated plasma samples for suPAR analyses were collected between 7:30–9:30 in the morning for two consecutive days after hospitalization and were frozen at -70 °C for future analyses. Plasma suPAR levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA, suPARnosticHStandard kit; ViroGates A/S, Birkerød, Denmark). Control samples were collected at the control visit at a median of 22 days (range 15–41) after the onset of fever and stored frozen until analysis.

4.2.8 Statistical methods (Studies I–IV)

For descriptive analyses, medians and ranges are given for continuous variables, and numbers and percentages for categorical variables. The Mann–Whitney U test was used to compare quantitative variables. Categorical data were analyzed by the Chi-square test or Fisher's exact test, as appropriate. Wilcoxon's test was used to compare two related samples, and the Kruskal–Wallis test was used when comparing three groups. Spearman's rank correlation was used to study correlations between variables. In Study I, logistic regression analysis was performed to identify factors determining the severity of AKI. Odds ratios (ORs) are expressed with their 95% confidence intervals (CIs). All tests were two-sided, and a p-value of <0.05 was considered statistically significant. For multiple tests, p-values were adjusted by Bonferroni correction. Statistical analyses were performed using the IBM SPSS software package (version 20 in Studies I and IV and version 27 in Studies II and III; IBM Corporation, Armonk, NY, USA).

4.3 Ethical considerations

All patients provided written informed consent to participate, and the Ethics Committee of Tampere University Hospital approved all studies (99256, R04180, R09206, R11188). The studies were conducted according to the principles of the Declaration of Helsinki. Routine patient care was not modified in any study.

5 RESULTS

5.1 Characteristics of the study population (Studies I–IV)

The clinical data and laboratory findings of the patients in Studies I–IV are presented in Table 7. The clinical presentation of all patients was typical of PUUV infection and did not differ between the studies. Transient dialysis treatment was needed in 19 patients in Study I, in one patient in Study II, in one patient in Study III (also included in Study I) and in five patients in Study IV (one included also in Studies I and II, and two patients included in Study I). On admission, clinical shock was present in seven patients in Study I, two patients in Study II, and none in Studies III and IV. One patient (included in both Studies I and II) suffered from Guillan-Barré syndrome probably triggered by acute PUUV infection. The patient was treated with plasma exchanges. All patients recovered.

Table 7. Clinical and laboratory findings of patients in Studies I–IV.

Clinical or laboratory finding median (range)	Study I N=357	Study II N=66	Study III N=27	Study IV N=97
Age (years)	40 (13-76)	41 (22-74)	41 (22-65)	41 (22-77)
Gender (men/women)	257/100	36/30	19/8	63/34
Change in weight during hospital care (kg)	2.5 (0-18.5)	2 (0-11)	2 (0-11)	2.1 (0-12)
Length of hospital stay (days)	7 (1-46)	6 (2-16)	6 (3-10)	6 (2-15)
Systolic blood pressure min (mmHg)	119 (60-170)	114 (74-170)	118 (74-144)	113 (74-170)
Diastolic blood pressure min (mmHg)	70 (36-100)	68 (40-89)	70 (40-89)	69 (40-100)
Creatinine max (µmol/L)	222 (51-1645)	175 (53-1148)	267 (60-1071)	175 (51-1499)
Cystatin C max (mg/L)	nm	1.9 (0.8-6.5)	2.2 (0.9-6.5)	nm
CRP max (mg/L)	70 (12-280)	91 (16-267)	92 (27-175)	85 (16-269)
Hematocrit min	0.36 (0.21-0.50)	0.36 (0.25-0.44)	0.35 (0.29-0.41)	0.36 (0.25-0.44)
Hematocrit max	0.43 (0.26-0.64)	0.44 (0.33-0.60)	0.46 (0.33-0.59)	0.44 (0.33-3.90)
Leukocyte max ($\times 10^9$/L)	9.8 (3.8-50.3)	10.5 (4.2-45.0)	12.3 (6.5-24.0)	10.4 (3.9-31.2)
Platelet count min ($\times 10^9$/L)	70 (9-378)	53 (5-150)	50 (15-133)	61 (9-187)

Abbreviations: max= maximum, min= minimum, CRP= C-reactive protein, nm=not measured.

5.2 Association of symptoms with the severity of infection (Studies II & III)

In Studies II and III patients' symptoms in relation to the clinical course of the acute illness were evaluated. In Study II the presence of abdominal pain, nausea, and vomiting was evaluated. In Study III the presence of back pain and cough was also reported. Almost all patients (23/27) in Study III were also included in Study II and therefore the presence of nausea/vomiting and abdominal pain are here presented as results from Study II. In Study II, 61% of the patients had nausea or vomiting and 27% had abdominal pain. Back pain was present in 67% and cough in 41% of the patients in Study III.

In Study II, patients with abdominal pain had higher maximum serum creatinine levels than patients without abdominal pain; median 350 μmol/L (range 76–1139) vs. 133 μmol/L (range 53–114s), p=0.020. Serum levels of cystatin C were not statistically higher in patients with and without abdominal pain; median 2.1 mg/L (range 1.2–6.5) vs. 1.6 mg/L (range 0.8–5.4), p=0.087. In Study II, patients with abdominal pain showed higher serum amylase concentrations than those without pain; median 66 U/L (range 33–134) vs. 40 U/L (range 13–181), p=0.006.

Table 8 presents clinical and laboratory findings in patients with various symptoms in Studies II and III. Also in this table, data from Study II on abdominal pain and nausea/vomiting are presented, because almost all the patients in Study III were also included in Study II.

Table 8. Clinical and laboratory findings in patients with various symptoms (Studies II and III). 23/27 patients in Study III were included in Study II.

	Abdominal pain Study II (N=66) Yes/No (18/48)	Nausea/vomiting Study II (N=66) Yes/No (40/26)	Back pain Study III (N=27) Yes/No (18/9)	Cough Study III (N=27) Yes/No (11/16)
Clinical findings				
Change in body weight (kg)	3.7 vs 1.7, **p=0.035**	2.3 vs 1.8, p=0.368	2.3 vs 1.7, p=0.463	1.5 vs 2.3, p=0.512
Length of hospital stay (days)	7 vs 6, p=0.286	6 vs 6.5, p=0.431	7 vs 6, p=0.940	6 vs 7, p=0.610
Systolic blood pressure min (mmHg)	121 vs 113, **p=0.041**	118 vs 108, **p=0.032**	120 vs 113, **p=0.031**	116 vs 119, p=0.904
Diastolic blood pressure min (mmHg)	69 vs 68, p=0.708	70 vs 67, p=0.315	71 vs 66, p=0.131	70 vs 68, p=0.716
Laboratory findings				
Creatinine max (µmol/L)	350 vs 133, **p=0.020**	179 vs 125, p=0.300	452 vs 83, **p=0.004**	148 vs 346, p=0.178
Cystatin C max (mg/L)	2.1 vs 1.6, p=0.087	1.9 vs 1.6, p=0.546	3.0 vs 1.5, **p=0.046**	1.9 vs 2.5, p=0.451
CRP max (mg/L)	82 vs 92, p=0.604	76 vs 106, **p=0.008**	88 vs 93, p=0.253	104 vs 84, p=0.318
Hematocrit max	0.44 vs 0.44, p=0.604	0.44 vs 0.44, p=0.942	0.47 vs 0.45, p=0.298	0.49 vs 0.45, p=0.148
Leukocyte count max ($\times 10^9$/L)	11.4 vs 10.0, p=0.154	11.4 vs 10.3, p=0.276	13.0 vs 9.0, p=0.053	12.3 vs 12.3, p=1.00
Platelet count min ($\times 10^9$/L)	74 vs 52, p=0.536	63 vs 50, p=0.084	47 vs 50, p=0.980	35 vs 52, p=0.064
Overnight urine albumin excretion (µg/min)	nm	nm	498 vs 423, p=0.781	396 vs 450, p=0.753

Abbreviations: min=minimum, max=maximum, CRP= C-reactive protein, nm= not measured.

5.3 Smoking and its association with the severity of infection (Studies I & II)

In Study I the association between cigarette smoking and the severity of AKI was evaluated in 357 patients. Altogether, 183 out of 357 (51%) patients were current

smokers at the onset of illness. One hundred eighteen (33%) patients had never smoked, and 56 (16%) were ex-smokers. Current smokers were younger than nonsmokers; median 38 vs. 45 years, $p<0.001$, and presented with fewer prior comorbidities than nonsmokers, 13% vs. 29%, $p<0.001$. Furthermore, current smokers suffered from cardiovascular diseases and/or diabetes less than nonsmokers, 6% vs. 16%, $p=0.001$. Men were more likely to be current smokers than women, 57% (147/257) vs. 36% (36/100), $p<0.001$.

In the analysis of non-respondents (n=155), smoking was even more prevalent in non-respondents than in the study group (73% vs. 49%, $p=0.018$). In this analysis, data on smoking habits was based on interviews by the research nurse during hospitalization.

The median maximum plasma creatinine level was significantly higher in current smokers compared with nonsmokers (273 vs. 184 μmol/L, $p<0.001$). Severe AKI was present in 75 of 183 (41%) current smokers vs. 46/174 (26%) of nonsmokers ($p=0.005$). To assess the effect of smoking cessation on the development of AKI, ex-smokers were compared with never-smokers. No significant differences were found in creatinine concentrations or other variables reflecting disease severity. Table 9 shows a comparison of clinical and laboratory findings between current smokers, ex-smokers, and never smokers.

Table 9. Comparison of clinical and laboratory findings between patients divided into three groups according to their smoking status.

			Nonsmokers						
	Current smoker (N=183)		Ex-smoker (N=56)		Never smoker (N=118)		p1	p2	p3
	Median	Range	Median	Range	Median	Range			
Change in body weight (kg)	2.7	0-16	2.8	0-8.7	2	0-18.5	0.125	0.104	0.455
Length of hospital stay (days)	7	1-27	8	3-17	7	2-46	0.553	0.41	0.533
Creatinine max (μmol/L)	273	51-1645	163	58-1281	197	52-1537	**<0.001**	**0.004**	0.563
CRP max (mg/L)	74	16-280	92	15-189	56	12-244	0.717	0.294	**0.003**
Hematocrit max	0.43	0.26-0.62	0.42	0.34-0.58	0.43	0.33-0.64	0.917	0.997	0.736
Leukocyte count max ($\times 10^9$/L)	10.8	4.1-44.7	8.8	4.2-31.2	9	3.8-50.3	**<0.001**	**0.001**	0.314
Platelet count min ($\times 10^9$/L)	67	9-378	73	24-246	70	10-314	0.181	0.243	0.983

Abbreviations: CRP= C-reactive protein.

p1=current smokers vs. nonsmokers, p2=current smokers vs. never smokers, p3=ex-smokers vs. never smokers.

This study did not show an association between the number of cigarettes smoked daily and plasma creatinine levels. Maximum creatinine levels did not differ in patients who smoked more than 20 cigarettes per day vs. patients who smoked 1–19 cigarettes daily (median 275 vs. 271 μmol/L, p=0.470). Neither did the leukocyte count differ between heavy smokers (>20 cigarettes/day) and those who smoked 1–19 cigarettes/day (median 10.6 vs. 10.8 $\times 10^9$/L, p=0.874). Leukocyte counts were significantly higher in current smokers compared with nonsmokers (median 10.8 vs. 8.9 $\times 10^9$/L, p<0.001). Leukocyte counts in ex-smokers and never smokers did not differ (median 8.8 vs. 9.0 $\times 10^9$/L, p=0.314). Maximum creatinine levels and maximum leukocyte counts showed a correlation (r=0.432, p<0.001).

In logistic regression analysis, smoking was the only independent factor influencing the risk of severe AKI (OR 1.8; 95% CI 1.1–3.0). Prior comorbidities, such as hypertension, diabetes, or atherosclerosis, did not influence the risk of AKI. There were no differences in maximum plasma creatinine levels between patients with and without these comorbidities (median 172 μmol/L, range 72–1026, vs. 226 μmol/L, range 51–1645, p=0.360).

Smoking status was also assessed in Study II. The patients were divided into current smokers and nonsmokers. Of alcohol drinkers, 14/17 (52%) were current smokers, and 22/39 (56%) of the abstainers smoked at the time of acute illness. Current smokers had higher median maximum serum creatinine and cystatin C concentrations than nonsmokers, but the p-values did not reach statistical significance: S-creatinine 207 μmol/L (range 53–1148) vs. 115 μmol/L (range 56–951), p=0.080, and S-cystatin C 2.1 mg/L (range 0.81–6.46) vs. 1.5 mg/L (range 0.84–4.67), p=0.050.

5.4 Liver enzymes and severity of infection (Study II)

Serum levels of ALT and GGT were measured during the period of hospital care one to five times and at the control visit. Levels of one or the other of the liver enzymes was slightly elevated during the acute phase of infection in 42/66 (64%) of the patients. Serum ALT concentrations were elevated in 24/66 (36%), and those of serum GGT in 35/66 (53%) patients. In three patients the upper limit of normal ALT levels was exceeded threefold, and in six patients the upper limit of normal GGT concentrations was exceeded threefold during the acute phase. Serum concentrations of neither of the liver enzymes correlated with the severity of AKI nor other variables reflecting the severity of the disease. Serum ALT and GGT levels were significantly higher at the acute phase compared with the convalescence phase (Table 10).

Table 10. Comparison of liver and pancreatic enzyme levels at the acute phase and the convalescence phase.

	Acute phase (N=66)			Convalescence phase (N=66)		
	Median	Range		Median	Range	p value
ALT max (U/L)	35	14-400		27	5-126	**0.003**
GGT max (U/L)	52	16-549		44	5-168	**0.007**
Amyl max (U/L)	48	13-181		74	20-215	**<0.001**
Trypsinogen max (ng/L)	6.6	1.6-33.7		1.6	1.5-11.1	**<0.001**
Trypsin/Crea max	1.1	0.1-14.9		0.2	0.1-3.5	**<0.001**

Abbreviations: max=maximum, ALT=alanine aminotransferase, GGT=gamma-glutamyl transferase, Amyl=amylase, Crea=creatinine, Trypsin/Crea=trypsinogen-creatinine ratio.

5.5 Alcohol consumption and severity of infection (Study II)

In Study II markers reflecting alcohol consumption were evaluated in 66 patients. Control-stage values were used in the analyses to avoid the confounding increase in liver enzyme levels due to acute infection.

Measures of GGT-CDT exceeded the cut-off value in 14% (5/36) of the men and 17% (5/30) of the women addressing heavy alcohol consumption. Seventeen patients showed U-EtG levels of more than 500 ng/mL, but their GGT-CDT values were under the cut-off limit and they were designated light drinkers. The rest (N=39) were classified as abstainers. No significant differences were found when these three groups were compared as regards clinical and laboratory variables. Table 11 shows the comparison of clinical and laboratory findings, and Table 12 shows a comparison of symptoms between these three groups. Altogether, 41% of the patients showed a biochemical sign of alcohol consumption before the control visit. Alcohol-consuming patients did not have symptoms more often than abstainers.

Table 11. Comparison of clinical variables and laboratory values between heavy drinkers, light drinkers, and abstainers.

	Use of alcohol			
	Heavy drinker (N=10)	Light drinker (N=17)	Abstainer (N=39)	p-value
Clinical finding				
Systolic blood pressure on admission (mmHg)	127 (83-138)	129 (72-161)	124 (86-182)	0.994
Diastolic blood pressure on admission (mmHg)	80 (65-100)	82 (40-110)	86 (52-107)	0.754
Length of hospital stay (days)	5 (3-9)	8 (2-15)	6 (2-12)	0.153
Change in body weight (kg)	1.6 (0.1-6.4)	3.5 (0.7-11.3)	1.8 (0-10.8)	0.447
Laboratory value				
ALT max (U/L)	33 (17-104)	34 (14-107)	35 (14-400)	0.967
GGT max (U/L)	98 (52-216)	49 (18-295)	50 (16-549)	**0.005**
Amyl max (U/L)	32 (13-68)	47 (22-147)	49 (18-181)	0.089
Creatinine max (µmol/L)	115 (56-1148)	172 (53-866)	200 (62-1139)	0.418
Cystatin C max (mg/L)	1.6 (0.9-4.9)	1.7 (0.8-5.0)	1.9 (0.8-6.5)	0.520
CRP max (mg/L)	99 (33-179)	99 (26-244)	82 (16-267)	0.266
Hematocrit max	0.41 (0.38-0.54)	0.44 (0.34-0.52)	0.45 (0.33-0.60)	0.123
Leukocyte count max ($x10^9$/L)	11.7 (4.2-18.3)	10.7 (8.5-18.9)	10.1 (6.4-45)	0.692
Platelet count min ($x10^9$/L)	78 (28-109)	60 (23-123)	43 (5-150)	0.142

The values are presented as medians and ranges. Max=maximum, min=minimum, ALT=alanine aminotransferase, GGT=gamma-glutamyl transferase, Amyl=amylase, CRP=C-reactive protein.

Table 12. Comparison of symptoms between heavy drinkers, light drinkers, and abstainers.

	Use of alcohol			
	Heavy drinker (N=10)	Light drinker (N=17)	Abstainer (N=39)	p-value
Symptom	N (%)	N (%)	N (%)	
Abdominal pain	1 (10%)	4 (24%)	13 (33%)	0.309
Nausea	5 (50%)	11 (65%)	22 (56%)	0.737
Vomiting	4 (40%)	9 (53%)	20 (51%)	0.785

Number of patients and percentages

When alcohol drinkers (including heavy and light drinkers) were compared with abstainers, no differences were found in the severity of AKI or variables reflecting

the severity of infection, such as length of hospital stay or change in body weight during hospital care. The median values of maximum serum creatinine and cystatin C did not differ between alcohol drinkers and abstainers at the acute phase: S-creatinine 118 μmol/L (range 53–1148) vs. 200 μmol/L (range 62–1139), p=0.208, and S-cystatin C 1.7 mg/L (range 0.8–5.0) vs. 1.9 mg/L (range 0.8–6.5), p=0.270, respectively. When heavy drinkers were compared with the others (light drinkers and abstainers), no differences were found in the severity of AKI, or levels of S-creatinine (115 μmol/L (range 56–1148) vs. 182 μmol/L (range 53–1139), p=0.325) or S-cystatin C (1.6 mg/L (range 0.9–4.93) vs. 1.9 mg/L (range 0.81–6.46), p=0.411).

Altogether, 19/66 patients (29%) had severe AKI (S-creatinine ≥353.6 μmol/L). Severe AKI was equally frequent among heavy alcohol drinkers (20%), light alcohol drinkers (24%), and abstainers (33%), p=0.607.

5.6 Pancreatic involvement in Puumala virus infection (Study II)

In Study II, patients with abdominal pain showed higher serum amylase concentrations than patients without pain (median 66 U/L (range 33–134) vs. 40 U/L (range 13–181), p=0.006). Serum amylase levels showed a correlation with serum creatinine and cystatin C concentrations (r=0.452, p<0.001 and r=0.403, p<0.001, respectively).

None of the patients met the criteria of acute pancreatitis. The highest measured serum amylase level at the acute phase was 181 U/L, and 94% (62/66) of the patients had serum amylase levels within reference values. Urinary trypsinogen levels were within normal limits in all patients. The highest measured value in this cohort was 33.7 ng/mL. Furthermore, the urinary trypsinogen/creatinine ratio was within normal limits in all patients.

The highest observed serum amylase level at the convalescence phase was 215 U/L. Altogether, serum amylase levels were significantly lower in the acute phase compared with the convalescence phase (median 48 U/L (range 13–181) vs. 74 U/L (range 20–215), p<0.001). Urinary trypsinogen levels and the trypsinogen/creatinine ratio, on the other hand, were higher in the acute phase compared with the convalescence phase (median 6.6 ng/L (range 1.6–33.7) vs. 1.6 ng/L (range 1.5–11.1), p<0.001, and median 1.1 (range 0.1–14.9) vs. 0.2 (range 0.1–3.5), p<0.001, respectively).

5.7 Associations between MRI findings and clinical severity and symptoms (Study III)

5.7.1 Fluid collections in the intraperitoneal, retroperitoneal and pleural space

Abdominal MRI was performed in 27 patients with acute PUUV infection at a median of six (range 3–11) days after the onset of fever. All patients showed fluid collections in MRI examination. Excess fluid was detected most frequently in the perirenal space (n=26), next to the psoas muscle (n=26), and in the Pouch of Douglas (n=26). Twenty-five patients demonstrated additional fluid in the pleural space. The amounts of fluid collections are presented in Table 13.

Table 13. Fluid accumulations in millimeters and number of patients presenting fluid collections.

	Perihepatic fluid (N=25)	Perisplenic fluid (N=19)	Fluid next to psoas muscle (N=26)	Pouch of Douglas fluid (N=26)	Perirenal fluid (N=26)	Pararenal fluid (N=21)	Pleural fluid (N=25)
Median	4	3	11	17	10	9	12
Minimum	0	0	0	0	0	0	0
Maximum	14	9	35	48	16	25	49

5.7.2 Fluid collections in relation to patients' symptoms

In Study III, 67% (18/27) of the patients suffered from back pain. Gastrointestinal symptoms were present in 18/27 (67%) patients, of which 17 (63%) were nausea or vomiting, and seven (26%) had abdominal pain. Patients with more severe fluid collections did not have symptoms more often than patients without symptoms. However, patients with back pain had less fluid in the perirenal space; 8 mm (range 0–14) vs. 13 mm (range 7–16), p=0.004.

5.7.3 Fluid collections in relation to clinical and laboratory findings

In Study III, different fluid collections in relation to the patients' clinical and laboratory findings were evaluated. The associations are presented in Table 14.

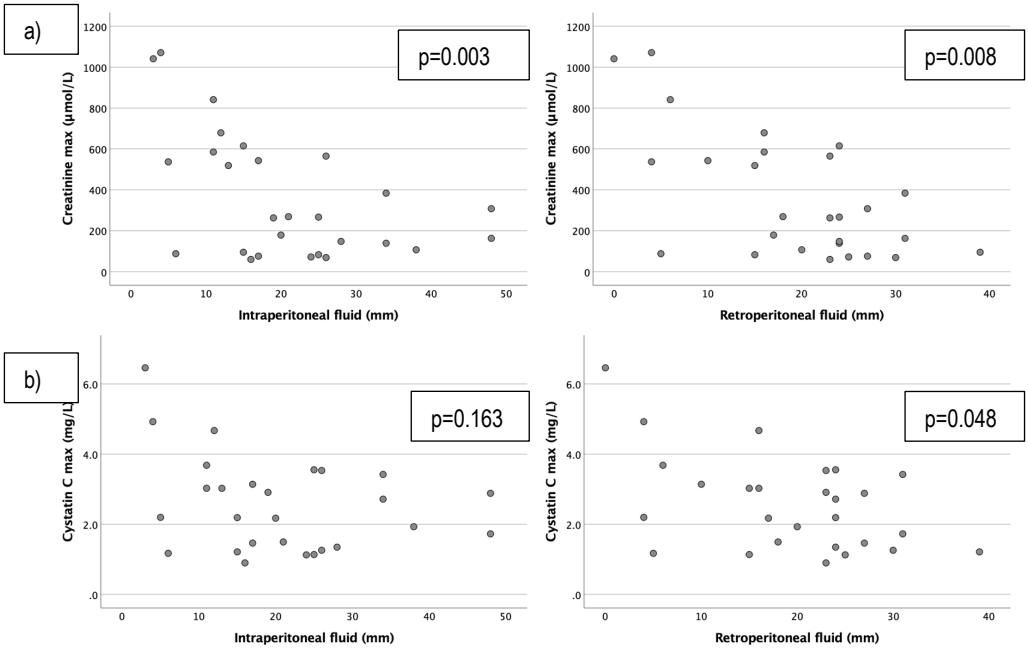
Intraperitoneal fluid accumulation correlated inversely with the change in body weight during hospital care. Both intraperitoneal and retroperitoneal fluid showed an inverse correlation with the minimum systolic blood pressure, and intraperitoneal fluid also with minimum diastolic blood pressure. Intraperitoneal fluid accumulation correlated positively with the maximum CRP level. Figure 2 shows the inverse correlation between plasma creatinine and serum cystatin C levels and the amount of intra- and retroperitoneal fluid.

Table 14. Associations of intraperitoneal, retroperitoneal, and pleural fluid with clinical and laboratory findings.

	Intraperitoneal fluid		Retroperitoneal fluid (peri- and pararenal)		Pleural fluid	
Clinical findings	r	P	r	P	r	P
Systolic blood pressure min (mmHg)	−0.773	**<0.001**	-0.596	**0.001**	0.277	0.162
Diastolic blood pressure min (mmHg)	-0.685	**<0.001**	-0.333	0.090	0.061	0.762
Change in body weight (kg)	-0.420	**0.029**	-0.375	0.054	0.436	**0.023**
Length of hospital stay (days)	-0.025	0.902	-0.022	0.914	0.093	0.645
Laboratory findings						
Creatinine max (µmol/L)	-0.418	**0.030**	-0.501	**0.008**	0.144	0.474
Cystatin C max (mg/L)	-0.277	0.163	-0.383	**0.048**	0.150	0.454
CRP max (mg/L)	0.586	**0.001**	0.328	0.095	0.085	0.675
Leukocyte count max ($\times 10^9$/L)	-0.048	0.813	-0.241	0.225	0.198	0.323
Hematocrit max	0.136	0.498	0.184	0.359	0.111	0.582
Platelet count min ($\times 10^9$/L)	-0.212	0.288	-0.153	0.445	-0.11	0.583
UAER (µg/minute)	0.072	0.720	-0.28	0.890	0.068	0.737

Abbreviations: max=maximum, min=minimum, CRP=C-Reactive Protein, UAER=Urinary Albumin Excretion Rate.

Figure 2. Correlations between a) maximum creatinine levels and b) maximum cystatin C levels with intraperitoneal fluid and retroperitoneal fluid.

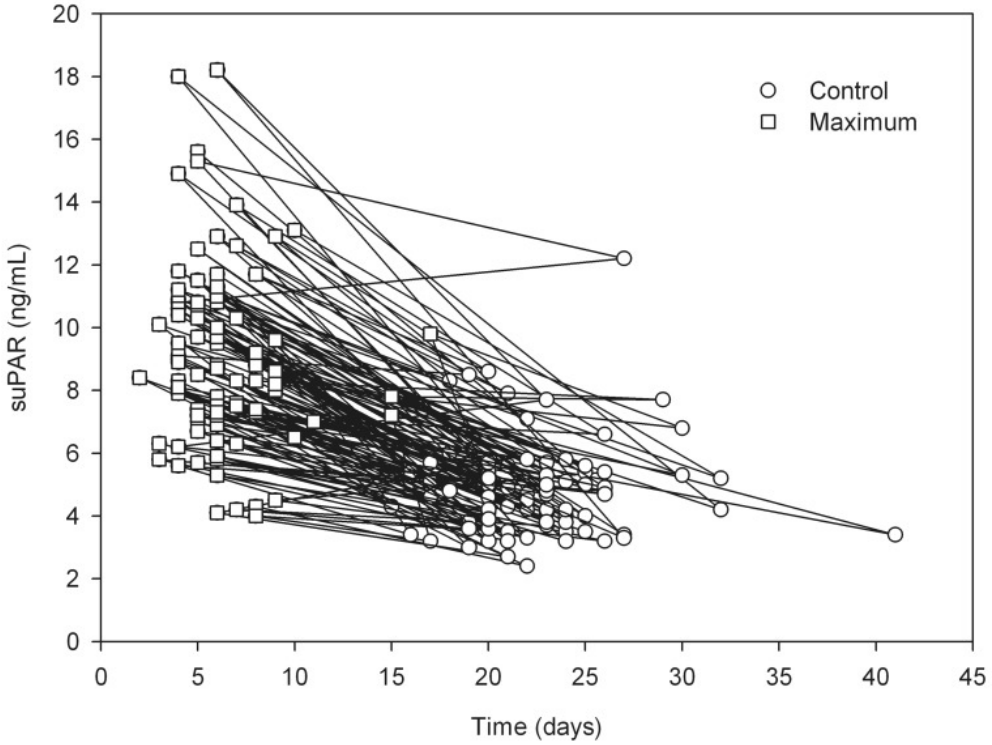


Abbreviations: max=maximum, mm=millimeters.

5.8 Association between soluble urokinase-type Plasminogen Activator Receptor and the severity of infection (Study IV)

Study IV evaluated the associations between plasma levels of suPAR and clinical and laboratory findings in 97 PUUV-infected patients. Acute-phase values were compared with control values taken at a median of 22 (range 15–41) days after the onset of fever in 84 (87%) patients. Maximum plasma suPAR levels were significantly higher at the acute phase of the disease compared with levels at the convalescence stage (median 8.7 ng/mL (range 4.0–18.2) vs. median 4.7 ng/mL (range 2.4–12.2), $p<0.001$). Figure 3 represents the maximum and the convalescence concentrations of suPAR in relation to the onset of fever.

Figure 3. Line chart showing plasma soluble urokinase-type plasminogen activator receptor (suPAR) maximum and control concentrations in relation to the onset of fever (day 0). Concentrations are presented as median values of the maximum and control values. (Adopted from Study IV, Outinen T. et al. "Plasma levels of soluble urokinase-type plasminogen activator receptor associate with the clinical severity of acute Puumala hantavirus infection. PLoSOne 2013;8(8):e71335).



SuPAR concentrations were associated with the severity of AKI. Plasma suPAR levels correlated with maximum creatinine concentrations, as shown in Table 15. Furthermore, patients who needed transient hemodialysis treatment (n=5) had higher suPAR concentrations than patients who did not need dialysis (median 10.8 ng/mL (range 8.6–18.0) vs. 8.4 ng/mL (range 4.0–18.2), p=0.038). In addition, plasma suPAR levels were associated with other laboratory markers reflecting the severity of PUUV infection, such as leukocyte count, IL-6 levels, PTX-3 and IDO concentrations, and cf-DNA levels. Table 15 shows the associations between suPAR levels and clinical and laboratory findings in the patients.

Table 15. Correlations between maximum plasma suPAR-levels and clinical and laboratory findings reflecting the severity of acute PUUV infection in 97 patients.

	r	p-value
Change in body weight during hospital care (kg)	0.406	**<0.001**
Length of hospital stay (days)	0.325	**0.001**
Urinary output min	-0.332	**0.002**
Creatinine max	0.378	**<0.001**
Hematocrit min	-0.369	**<0.001**
Leukocyte count max	0.475	**<0.001**
Platelet count min	-0.325	**0.001**
CRP max	0.298	**0.003**
cf-DNA max	0.363	**0.018**
IDO max	0.557	**<0.001**
IL-6 max	0.621	**<0.001**
PTX3 max	0.425	**0.005**

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

6 DISCUSSION

6.1 Study population

This study was conducted at Tampere University Hospital, and all patients were hospitalized as a result of PUUV infection. Owing to this bias toward more severe infections, strong conclusions can only be drawn in connection with hospital-treated patients. The data-collection period was notably long, thirty-two years. The methodology used in the diagnosis of PUUV infection changed during this time, from an IgG antibody increase-based method to ELISA [83], nevertheless, both methods are reliable. There is no specific treatment for PUUV infection, and the basis of supportive treatments has been similar throughout the years.

Patients in all studies were relatively young and healthy, and the incidence of comorbidity did not differ between the four studies. All studies partly included the same patients. Nevertheless, the total number of 547 patients is considerable.

6.2 Association between symptoms and the severity of infection

Fever, and abdominal and back pain are often reported in acute PUUV infection [5–9]. The exact reason for abdominal pain still has no explanation. Patients' symptoms were reported in Study II and Study III. Since almost all patients in Study III were also included in Study II, the association of abdominal pain with clinical and laboratory findings in Study II was reported. Abdominal pain was present in 27% of patients in Study II, a finding which is in concordance with earlier studies [5–9]. Patients presenting with abdominal pain had more severe AKI (Study II).

The presence of back pain was evaluated only in Study III, and it was associated with more severe AKI. Flank/back pain is also frequently reported in other forms of acute tubulointerstitial nephritis, and it is considered to originate from renal capsule stretching [226,227]. Stretching is thought to be a result of inflammatory cells infiltrating into the interstitium [226,227]. Kidney swelling is also seen in acute pyelonephritis resulting from inflammatory edema and cellular infiltration [228]. In PUUV infection, inflammatory cells, such as lymphocytes, infiltrate into the kidneys

[12,63,229]. In addition, hemorrhages are common and may also stretch the renal capsule, causing pain. Pain sensation may vary between patients and sometimes it is difficult to distinguish whether the pain originates from the abdomen or the back/flank. Therefore, abdominal pain may also indicate, at least partly, renal capsule stretching. Patients with more severe AKI had back pain more often, which may result from more abundant infiltration of inflammatory cells into the kidney, contributing to more intense capsule stretching.

In Study II, serum amylase concentrations were higher in patients with abdominal pain, but none of the patients showed amylase concentrations three times over the upper limit of normal, which would suggest acute pancreatitis. Serum amylase is eliminated through the kidneys, and elevated levels may reflect the degree of oliguric AKI and diminished elimination. Serum amylase levels showed a correlation with creatinine and cystatin C concentrations, supporting this.

In conclusion, back pain and abdominal pain in acute PUUV infection may result from capsule stretching due to an enhanced number of inflammatory cells and hemorrhages. Back pain and abdominal pain seem to be associated with more severe AKI.

6.3 Association between cigarette smoking and the severity of infection

In Study I, cigarette smoking was shown to aggravate acute kidney injury in PUUV-infected patients, but smoking cessation returned the risk of severe AKI to the same level as in never-smokers. Previous studies have shown cigarette smoking to be an independent risk factor of PUUV infection [20,21]. With the design of the present study, the OR of acquiring acute PUUV infection is impossible to assess, but patients smoked clearly more often than the general population, supporting the results of earlier studies. Unexpectedly, current smokers had fewer comorbidities than nonsmokers. They were also younger than nonsmokers, which might at least partly explain the finding.

In Study I, data on smoking history was collected via a questionnaire. Non-respondents were compared with respondents to evaluate the reliability of the data. Non-respondents were younger and even more often current smokers than responders. The smoking history of non-respondents was based on interviews by the research nurse during the hospital stay. In that respect, the present study does not overestimate the number of current smokers. Otherwise, there were no

differences between the respondents and non-respondents in gender distribution, body mass index or clinical severity of PUUV infection, and patients who took part in the study represented the entire study population well.

Cigarette smoking results in worsened outcome in various infectious diseases [19,188,192]. Recently, smoking has been shown to be a risk factor of AKI in patients with COVID-19 infection [230]. In Study I, current smokers had more severe AKI than nonsmokers. In addition, severe AKI, according to KDIGO practical guidelines (a serum creatinine level equal to or more than 353.6 μmol/L), was more prevalent in smokers than in nonsmokers. On the other hand, ex-smokers did not have more severe AKI than never-smokers, reflecting the reversibility of the adverse effects of smoking. Advanced age and pre-existing CKD are well-known risk factors of AKI [231]. In Study I, current smokers were, however, younger than nonsmokers, and none of the patients had any CKD prior to PUUV infection.

Blood leukocyte counts were also higher in current smokers than in nonsmokers. Cigarette smoking is known to increase peripheral blood leukocyte counts by 30%, which may be due to generalized stimulation of the bone marrow [18]. Higher leukocyte counts in current smokers may therefore reflect both cigarette smoking itself, and a more severe PUUV infection, as maximum leukocyte counts and maximum creatinine levels also showed a correlation.

Cigarette smoking is an independent risk factor of CKD [232], and smoking exacerbates kidney disease progression [197]. The effects of smoking on the development of AKI are far less well studied. In both acute and chronic kidney injury, smoking has hemodynamic and non-hemodynamic effects on the kidney. Smoking leads to increases in blood pressure and heart rate, mainly because of nicotine [233]. In the present study, blood pressure did not differ between current smokers and nonsmokers, suggesting that non-hemodynamic mechanisms may be more involved with PUUV-induced AKI.

Non-hemodynamic effects of CS on the progression of CKD are heavy metals, hypoxia, prothrombotic factors, oxidative stress, proinflammatory cytokines, and activation of nicotine acetylcholine receptors [233]. Information on these effects in AKI is limited. Smoking is known to increase oxidative stress in various organs including the kidneys. Mitochondria are also affected by cigarette smoke. Arany and coworkers have shown in a mouse model that chronic nicotine exposure increases protein p66shp expression, resulting in mitochondrial ROS production, thus leading to injury of cultured proximal tubular cells and aggravated AKI [198]. Exposure to chronic levels of cigarette smoke or nicotine leads to morphological changes in the proximal tubular epithelium [234]. Furthermore, low-grade damage in the proximal

tubules has been found among smokers in the general population [235,236]. In acute PUUV infection, a characteristic histopathological finding is tubulointerstitial nephritis. The pre-existing morphological changes in smokers' renal tubular cells may contribute to more severe tubulointerstitial nephritis in PUUV infection. In addition, smoking can lead to endothelial dysfunction [233]. Further, hantaviruses infect endothelial cells, leading to dysfunction of the normal endothelial barrier. Taking these commonalities into account, it seems plausible that there might be differences in the endothelial function of smokers and nonsmokers at the time of PUUV infection, resulting in different outcomes of renal injury.

A questionnaire-based study design has limitations. Nevertheless, when the validity of the questionnaire was assessed by comparing the data from the questionnaire with interview-based data, there was no inconsistency. Furthermore, previous studies have shown that the validity of self-reported smoking history is good, and smokers rarely claim to be nonsmokers [237–239]. The results of Study I were confirmed in a German study a year later [240]. The investigators performed a prospective cross-sectional survey of 485 German patients with previous PUUV infection, of whom 61% had data on smoking history available. Altogether, 34% of the patients were current smokers, and the peak creatinine concentration was significantly higher among smokers compared with nonsmokers [240]. The German study confirmed the adverse effect of cigarette smoking on PUUV-induced AKI.

In Study II, smoking status was assessed in addition to alcohol consumption. As in Study I, active smokers were also over-represented in Study II compared with the general population. Of 66 patients, 55% were active smokers. Serum creatinine concentrations were higher in active smokers than in nonsmokers, but the difference was statistically insignificant. The insignificant result might be due to a smaller sample than in Study I. Nevertheless, the results in Study II are in line with those in Study I.

In conclusion, smoking is more common in hospitalized patients with acute PUUV infection than in the general population. Current smokers are at increased risk of suffering more severe PUUV-induced AKI. Nevertheless, the effect of CS seems to be reversible, as smoking cessation decreases the risk to the same level as in never-smokers.

6.4 Association between liver enzymes and the severity of infection

The clinical relevance of slightly elevated levels of liver enzymes during hantavirus infection is uncertain. One study carried out in Greece concerned liver involvement in hantavirus infection [74]. Nine out of 32 patients had liver involvement. Of these, 4/9 patients (44%) died, compared with patients without liver involvement, of whom 3/23 (13%) died. In that study, liver involvement was more frequent in patients with thrombocytopenia and more severe AKI [74]. A contrasting finding was made in a Finnish study, in which patients with severe AKI did not have higher serum ALT levels than patients with milder AKI [82]. The discrepancy may be the result of different causative agents, i.e., PUUV in Finland and probably DOBV in Greece. The overall fatality rate in cases of DOBV infection is higher than in PUUV infection [36]. In Study II, neither GGT nor ALT concentrations correlated with the severity of AKI or other variables reflecting overall disease severity. PUUV may cause inflammation of the liver. PUUV has been detected in the lymphocytes of hepatic sinusoids [134]. However, there are no studies showing PUUV in hepatocytes. In clinical settings, it is common to detect slightly elevated levels of liver enzymes during acute infection, and normalization of these levels usually appears without intervention. This phenomenon is most likely due to inflammation of the liver.

In conclusion, levels of liver enzymes are elevated during acute PUUV infection, but they are not associated with the severity of PUUV-induced AKI or the severity of the infection in general.

6.5 Association between alcohol consumption and the severity of infection

In Study II, biomarker-based evaluation of alcohol consumption showed that it was relatively common among the patients. Nevertheless, alcohol consumption did not seem to affect the clinical course of acute PUUV infection. Considering the possible confounding effect of an acute infection on serum GGT levels, control-phase values were used to assess patients' alcohol consumption. The sensitivity of GGT to detect heavy alcohol consumption is 70–73%, but a variety of other conditions, such as obesity, smoking, and drug use, may alter the concentrations [210,211]. Another widely used marker of alcohol consumption is CDT, which has good specificity but

relatively low sensitivity. The levels of both GGT and CDT normalize on average within two weeks after the cessation of alcohol use [27]. The combination marker GGT-CDT has been shown to improve sensitivity and specificity in detecting heavy alcohol consumption. In Study II, this marker was assessed in 66 patients. It showed heavy alcohol consumption in 14% of men and 17% of women. This is clearly more often than has been reported earlier in the Finnish population. In the FINRISK study of 13 976 individuals, the prevalence of heavy alcohol consumption among the general population was 4.2% [241]. In that study, a heavy drinker was defined as a person consuming more than 276 grams (men) or 180 grams (women) of alcohol per week [241]. GGT-CDT has been proposed to react when regular alcohol intake exceeds 40 g per day [216], which makes the definition of a heavy user of alcohol congruent between the present study and the FINRISK study. However, the FINRISK study is based on self-reported amounts of alcohol used, which may underestimate the actual consumption. Therefore, there might be a bias toward lesser consumption as compared with the biomarker-based approach. Nevertheless, the results of the present study indicated a more than threefold greater prevalence of heavy alcohol consumption in patients with acute PUUV infection compared with that in the general population, which the self-reported bias cannot fully explain.

When assessing alcohol intake, specific ethanol metabolites can be used. EtG is conjugated in the liver and can be detected in urine up to 90 hours after ethanol intake [219]. It is possible that U-EtG shows a positive result after a single instance of alcohol intake. Therefore, it cannot be used as an indicator of chronic alcohol abuse. Levels of U-EtG may also rise after using alcohol-containing cosmetics and hand disinfectants [218]. With a cut-off limit of 500 ng/mL, these other sources of ethanol exposure can be excluded. In the present study, there was only one patient whose U-EtG concentration was over 500 ng/mL on admission to the hospital. Patients sought medical help at a median of four days after the onset of fever. Since the half-life of EtG is short, the concentrations measured at the acute phase reflect alcohol use during the febrile phase of the illness. Therefore, it seems evident that consumption of alcoholic beverages was less than usual among the patients. At the control phase, 30% of the patients showed U-EtG concentrations over 500 ng/mL, indicating recent alcohol use. Since the control samples were taken at a median of 24 days after the onset of fever, it can be assumed that most of the patients were fully recovered at that time.

Patients who showed U-EtG concentrations of more than 500 ng/nL at the control visit but who did not exceed the GGT-CDT cut-off limit were designated as light drinkers. These patients showed some biochemical signs of alcohol use but did

not fulfill the criteria of a heavy user. A patient was designated as an abstainer when neither of the alcohol biomarkers (GGT-CDT or EtG) was elevated. Fifty-nine percent of the patients fulfilled these criteria. The number of true abstainers, who never use alcoholic beverages, is presumably much smaller, because U-EtG reflects alcohol consumption for only up to five days before sampling [216]. In the FINRISK study, based on interview data, 34% of the respondents reported themselves to be abstainers [241].

A limitation in Study II is the lack of the most accurate laboratory test to show recent alcohol consumption, namely assay of phosphatidylethanol (PEth). PEth is only formed in the presence of ethanol, and therefore there are no false-positive results. In addition, the sensitivity is very high, reaching 99% [223]. PEth measurement requires fresh blood samples for detection in red cells, and therefore it was not possible in this study. Nevertheless, the combination marker GGT-CDT is only a little behind in sensitivity compared with PEth. Mean corpuscular volume (MCV) was not evaluated either in this study. Since the normalization of elevated MCV values after drinking cessation takes 2–4 months, the additional information in respect of this study setting would have been little. Furthermore, another limitation is the omission of a questionnaire-based interview, such as the Alcohol Use Disorders Identification Test (AUDIT). Biochemical tests for alcohol consumption were carried out at the control visit. It was hypothesized that alcohol consumption was at the patients' regular level at that time, but since the interview data is lacking, it remains an assumption.

In Study I, smoking was associated with more severe AKI. There are several reports on active smoking being associated with alcohol drinking [22–24]. In addition to smoking, alcohol consumption affects the immune system. Chronic alcohol abuse alters the immune system in various ways, and chronic alcohol consumption predisposes individuals to several infectious diseases. In Study II, alcohol drinkers were compared with abstainers in respect of the clinical course of the disease. Alcohol drinkers did not have more severe AKI than abstainers. Furthermore, when alcohol drinkers were divided into heavy drinkers and light drinkers, no differences in serum maximum creatinine or cystatin C concentrations were found as compared with abstainers. The proportions of patients with severe AKI did not differ between these three groups. Other variables reflecting the severity of acute infection, such as length of hospital stay, change in body weight during hospital care, maximum leukocyte count, and minimum platelet count, did not differ between alcohol drinkers and abstainers.

Previous studies have implied that chronic alcohol exposure worsens the clinical outcome in Listeria monocytogenes infection and in various pulmonary infections [25]. Of viral diseases, hepatitides are most studied in relation to alcohol exposure. Alcohol worsens the clinical outcome of hepatitis B, hepatitis C, and cytomegalovirus-induced hepatitis [25]. No previous data on the effects of ethanol exposure on the clinical outcome of any hantavirus disease has been published. Nevertheless, alcohol does not lead to a more severe course of PUUV infection, but it may be a predisposing factor as regards hantavirus infection. In the present study, heavy consumption of alcohol was more prevalent among PUUV-infected patients as compared with data from an earlier survey of the general population [241]. Alcohol heavy consumption may therefore increase the susceptibility to PUUV infection, but this finding needs to be confirmed in a more extensive material and a different kind of study setting.

In conclusion, Study II showed biomarker-based signs of alcohol use in 41% of the patients at the control visit. Alcohol consumption did not seem to affect the clinical outcome of an acute PUUV infection.

6.6 Pancreatic involvement in acute Puumala virus infection

In addition to liver enzymes and alcohol biomarkers, Study II evaluated pancreatic involvement in acute PUUV infection. Conflicting results concerning hantavirus-induced acute pancreatitis have been established worldwide. There are several reports on HFRS accompanied by acute pancreatitis [242–248]. The causative agents in these studies have been Hantaan, Dobrava and Seoul viruses. The definition of acute pancreatitis in the present study was the presence of abdominal pain and a threefold elevation of serum amylase levels. None of the patients fulfilled both criteria. In addition, urinary trypsinogen gives 100% specificity for acute pancreatitis with a cut-off limit of 100 ng/mL [249], and none of the patients exceeded that limit. According to guidelines, a typical imaging finding for pancreatitis is also included in the criteria, but such imaging findings were not available. There are also other studies supporting our findings. Kitterer and coworkers studied 166 patients with acute PUUV infection. They found no acute pancreatitis in abdominal ultrasound examination or computed tomography (CT) imaging [76]. Recently CT imaging of 30 patients with acute PUUV infection was evaluated, and no signs of acute pancreatitis were revealed [17]. Conflicting findings probably arise from the different

causative agents such as HTNV, DOBV and SEOV leading to more severe infection in general, which may therefore affect several organs, including the pancreas.

Serum amylase concentrations were significantly lower during the acute phase of the disease compared with the convalescence stage. This finding is exciting and indicates a need of future studies in more extensive materials. One explanation could be that patients had been nauseous and lost appetite before admission to hospital. Due to their low nutrient intake, patients may have secreted lower amounts of salivary and total amylase. This might at least partly explain why instead, levels of urinary trypsinogen were higher at the acute phase than in the control phase. Trypsinogen is secreted only from the pancreas and not the salivary glands.

In conclusion, Study II showed no acute PUUV-induced pancreatitis. Serum amylase concentrations were lower at the acute stage as compared with the convalescence stage.

6.7 Association between fluid collections and the severity of infection

In Study III, fluid collections were found in all patients in abdominal MRI. Retroperitoneal or intraperitoneal fluid collections, except small amounts of fluid in fossa Douglas in females, are not usually found by means of imaging in healthy individuals [250]. In a recent Belgian study, perirenal or retroperitoneal fat stranding and/or perirenal fascial thickening were found in most patients with acute PUUV infection [17]. Pelvic ascites was found in 50% of the patients [17]. The patients had been examined by means of CT imaging. It is less accurate than MRI, especially without contrast enhancement. Differentiation between a small amount of fluid and bordering soft tissue structures in CT is limited to identification of fascial thickening and fat stranding, which both represent non-measurable fluid accumulations. In that respect, the Belgian study is in line with the present study demonstrating fluid collections in all studied patients.

Fluid collections were evaluated in relation to the patients' symptoms. Sixty-seven percent of the patients suffered from back pain, which is in line with the results of earlier studies [5–9]. An interesting finding was that a higher amount of fluid did not increase patients' symptoms. In contrast, patients with back pain had less fluid in the abdomen, especially in the perirenal space, than patients without back pain. In earlier literature, the reason for abdominal and back pain during acute PUUV infection has been proposed to be retroperitoneal edema due to increased capillary leakage

[37,251]. Supporting evidence is lacking, and the results of Study III indicate a different picture. It is plausible that back pain in PUUV infection results from renal intracapsular processes and capsular extension rather than fluid accumulation.

An inverse correlation emerged between the amount of retroperitoneal fluid and serum maximum creatinine and cystatin C concentrations. In addition, there was an inverse correlation between the amount of intraperitoneal fluid and change in body weight. During acute PUUV infection body weight may vary greatly owing to a change between oliguric and polyuric stages and patients also often receive intravenous fluid during their hospital care. It has been speculated that the change in body weight might reflect the degree of vascular leakage [65]. In Study III the severity of AKI and change in body weight during hospital care correlated. Because the amount of intraperitoneal and retroperitoneal fluid correlated inversely with the degree of AKI, the results of Study III suggest that the change in body weight during acute PUUV infection reflect the degree of AKI and not capillary leakage. AKI development and increased vascular leakage seem to proceed independently from each other. Plasma CRP is a marker of inflammation, and it showed a positive correlation with intraperitoneal fluid. Intraperitoneal and retroperitoneal fluid correlated inversely with minimum systolic blood pressure, and intraperitoneal fluid also with minimum diastolic blood pressure. These results may indicate increased capillary leakage due to inflammation.

In Study III, pleural fluid collections were found in most patients. Pleural fluid was present more often than in the Belgian study [17], but the difference may be explained by the better sensitivity of MRI to detect small amounts of fluid as compared with CT imaging. A previous Finnish study showed a correlation between pulmonary findings and the severity of AKI [156]. The present study did not show such an association. The conflicting results may be due to differences in imaging modalities. Abdominal MR imaging reached only the basal parts of the lung and in the present study the parenchymal changes in the lung were not evaluated. This may at least partly explain why there was no correlation between pulmonary findings and the severity of AKI.

The relatively small sample size in Study III is a limitation as regards drawing strong conclusions from the results because of the large standard error. There is one previous study on renal MRI findings and their clinical correlation in acute PUUV infection [252]. In a Finnish study by Paakkala and coworkers, fluid collections were found in 80% of the patients, but the amount of fluid was assessed only qualitatively. Associations between fluid collections and clinical variables did not reach statistical significance, but patients with fluid collections had slightly lower maximum

creatinine concentrations and higher CRP levels [252], findings which are in line with those in Study III. The obvious strength of Study III is the precise evaluation of locations and sizes of fluid collections during acute PUUV infection. Since the measurements were made quantitatively the study will easily be reproducible in more extensive materials.

In conclusion, fluid collections are present in acute PUUV infection. Increased fluid amounts, however, do not reflect more severe AKI. Instead, they may appear as a protective sign as regards the development of a more severe AKI. On the other hand, fluid collections appear to be associated with the degree of inflammation and capillary leakage, which seem to proceed independently of AKI. It is likely that a change in body weight during hospitalization results from changes between oliguria and polyuria and does not reflect the degree of vascular leakage. Finally, back pain during acute PUUV infection is more prevalent in patients with more severe AKI.

6.8 Association between soluble urokinase-type Plasminogen Activator Receptor levels with the severity of Puumala virus infection

In Study IV, suPAR concentrations were measured during acute PUUV infection and at the control visit. SuPAR is proposed to predict the course of the disease in various conditions. At the time when Study IV was conducted, there was only a little information available on suPAR as a predictive biomarker in viral diseases. Since then, several studies have been published and understanding has widened.

Study IV showed significantly higher maximum suPAR concentrations during hospital care compared with levels in samples taken at the control visit. Control samples were taken at a median of 22 days after the onset of fever and all patients were fully recovered at that time. The median control value of 4.7 ng/mL is in line with earlier reported values among the general population [253]. Recently, plasma/serum suPAR concentrations have been studied in several bacterial infections. In a meta-analysis of 17 studies, suPAR was evaluated in nine studies with 1237 patients as a marker of bacterial infection, such as sepsis, with a sensitivity of 0.73 and specificity of 0.79 [143]. In the same meta-analysis a pooled risk ratio of 3.37 (95% CI 2.60–4.38) was reported for risk of death [143]. In addition, suPAR has shown increased levels and has predicted mortality in tuberculosis, malaria, and purulent meningitis [145,146,154]. SuPAR has been studied in connection with several viral infections: hepatitis B and C, HIV-1, CCHF and most recently in

COVID-19 [148,155,254–257]. In these studies, higher suPAR concentrations were associated with a more severe outcome of the infection.

In Study IV, the maximum plasma suPAR concentration correlated with several variables reflecting PUUV infection severity, i.e., maximum serum creatinine concentration, length of hospital stay, change in body weight and maximum blood leukocyte count. Plasma suPAR showed an inverse correlation with minimum platelet level and minimum urinary output. Further, the maximum plasma suPAR concentration was significantly higher among patients who needed transient hemodialysis treatment. This association of higher plasma suPAR concentrations and a more severe course of PUUV infection is in line with the results of other studies showing similar associations [145–151,155]. Plasma suPAR levels also correlated with earlier studied biomarkers predicting the outcome of PUUV infection, i.e., PTX-3, IL-6, IDO, and cf-DNA [28–31]. PTX3, IL-6 and IDO reflect activation of the immune system in addition to the severity of PUUV infection. SuPAR has also been proposed to act as a biomarker of systemic chronic inflammation [258].

A positive correlation between maximum plasma suPAR levels and CRP concentrations was found in Study IV. An earlier Finnish study has indicated that higher CRP levels do not predict more severe kidney injury in PUUV-induced AKI [30]. An opposite finding was reported later in a German study [77]. The investigators found an association between a high CRP level and more severe AKI [77]. Nevertheless, CRP and suPAR concentrations might have reflected immune activation and inflammation in the present study. In addition to serving as a biomarker of systemic inflammation, suPAR has been shown to predict cancer, type-2 diabetes, cardiovascular disease, and mortality in the general population [253]. SuPAR is known to be of prognostic value in coronary artery disease and to some extent in heart failure also [259,260].

SuPAR has been proposed to play a pathogenic role in the development of chronic kidney disease. First, it was reported to interact with an αvβ3 integrin on kidney podocytes in focal segmental glomerulosclerosis (FSGS), resulting in foot process effacement and progression of proteinuria [261]. This finding still remains uncertain as later Harel and coworkers demonstrated that suPAR by itself does not injure podocytes in a mouse model [262]. There are also other studies challenging the theory of suPAR contributing to the pathogenesis of FSGS [263–266]. Nevertheless, higher concentrations of suPAR are seen in patients with FSGS as compared with the healthy population. As regards hantaviral diseases, the interaction of suPAR with $\alpha_v\beta_3$ integrin seems interesting, as pathogenic hantaviruses use β3

integrins in cellular entry [267]. In a study by Outinen et al. urinary suPAR concentrations were significantly higher at the acute stage of PUUV infection as compared with the convalescence stage, but plasma suPAR levels and those of urinary suPAR did not correlate [268], while urinary suPAR concentrations correlated with the degree of proteinuria. It seems plausible that there is local production of suPAR in the kidney.

In conclusion, plasma suPAR concentrations are elevated in acute PUUV infection and are associated with a more severe course of the illness. In addition to the severity of AKI, suPAR may also reflect activation of the immune system. Circulating suPAR may also contribute to the pathogenesis of PUUV infection by activating β3 integrins.

6.9 Future considerations

Despite the expanding lines of studies carried out in attempts to reveal the complete pathogenesis of hantaviral diseases, it remains only partly understood. Hantaviral diseases are emerging infections globally. Though PUUV infection usually presents as a mild course of events, effective treatment, and prevention of more severe hantaviral diseases are eagerly sought. The interactions between hantaviruses, β3-integrins, and suPAR are fascinating because they are linked in the pathogenesis of hantaviral diseases. Understanding the pathogenesis completely could lead to the development of therapies. Study III showed an interesting negative correlation between the amount of the fluid in the abdomen and the degree of AKI, a finding which was surprising and against the primary hypothesis. Since the amount of abdominal fluid correlated with CRP concentrations, the results of Study III suggest that capillary leakage contributes to increased inflammation. Therefore, it would also be of interest to study pro-inflammatory cytokines in relation to fluid collections. The results of Study III need to be confirmed in more extensive material. Since abdominal ultrasonography is a relatively cheap and noninvasive examination, it could be used instead of MRI. The correlations between fluid collections and patients' clinical and laboratory findings should be explored in larger study populations.

The adverse events of cigarette smoking are widely known. Nevertheless, the effects of CS on the development of AKI are still not elucidated. Preliminary animal studies have been conducted [198,234], but large cohort studies on humans are still lacking. AKI is a common comorbid condition in severe infections [269]. Since Study

I showed that cigarette smoking significantly increased the risk of severe AKI, and the German study [240] confirmed this finding, it would be interesting to further investigate the association between smoking and AKI in other infectious diseases. A large cohort study in the United Kingdom concerned risk factors of developing AKI among elderly patients with diabetes and community-acquired pneumonia [270]. Current cigarette smoking did not increase the risk of AKI in that study [270]. The conclusion that smoking is not a risk factor of infection-induced AKI is somewhat conflicting, since there are studies showing that there is such a relationship in, for example, COVID-19 and sepsis [271,272]. A large Finnish register, FINNDATA, could provide materials for future studies of the association between smoking and infection-related AKI.

7 SUMMARY AND CONCLUSIONS

The main findings of the studies lead to the following conclusions:

1) Active cigarette smoking is associated with more severe AKI in patients hospitalized due to acute PUUV infection. Nevertheless, the adverse effect of cigarette smoking seems to be reversible, as ex-smokers do not have such a risk. Active cigarette smoking is more prevalent in hospitalized PUUV-infected patients as compared with the general population.

2) Alcohol consumption does not seem to be associated with more severe PUUV-induced AKI, nor other variables reflecting disease severity, such as length of hospital stay, change in body weight during the hospital stay, or the minimum platelet count. Elevated levels of liver enzymes are often present during acute PUUV infection but are not associated with the severity of the disease.

3) Acute pancreatitis is not present in PUUV-induced AKI. Serum amylase concentrations were significantly lower during acute infection as compared with control values. Higher amylase levels at the acute stage were associated with abdominal pain.

4) Fluid collections were found in all patients studied by means of MRI. The amount of fluid did not reflect more severe AKI. Instead, there were inverse correlations between the amounts of intraperitoneal and retroperitoneal fluid, and serum creatinine concentrations. Capillary leakage and AKI seem to proceed independently during the acute phase of PUUV infection. Back pain is associated with more severe AKI and seems to originate from intracapsular processes and renal capsule stretching.

5) Plasma suPAR levels are elevated during the acute phase of PUUV infection. SuPAR levels are associated with the severity of AKI and are higher in patients who require transient dialysis treatment.

In conclusion, of lifestyle-related factors, cigarette smoking is related to more severe PUUV-induced AKI. Smoking cessation returns the risk to the same level as in never-smokers. Even though alcohol use alters the immune system in various ways, it does not seem to lead to a more severe outcome in PUUV infection.

Fluid collections are ubiquitous during acute PUUV infection, but the amount of fluid is not associated with more severe AKI. The accumulation of fluid may be a result of increased inflammation and capillary leakage. It seems that AKI and capillary leakage proceed independently of each other during acute PUUV infection. Since plasma suPAR levels are associated with more severe AKI and also with markers reflecting capillary leakage, suPAR in PUUV infection may indicate both renal injury and capillary leakage.

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APPENDIX 1

Myyräkuume ja elämäntavat -kysely 02.01.2012

Nimi _____Syntymäaika _____

Kysymme tupakointitottumuksianne **silloin, kun olitte sairaalahoidossa myyräkuumeen vuoksi _____sekä ajalta ennen sairastumista**.

1) Olitteko tupakoitsija, kun tulitte hoitoon Tampereen yliopistolliseen sairaalaan myyräkuumeen vuoksi?

1. Kyllä
2. En ollut silloin tupakoitsija, mutta olin tupakoinut aiemmin. Siirtykää kysymykseen 3.
3. En ollut koskaan tupakoinut ennen sairastumistani myyräkuumeeseen. Siirtykää kysymykseen 5.

2) Jos olitte tupakoitsija silloin, kuinka paljon poltitte keskimäärin päivässä?

1. Yli 20 savuketta, sikaria tai piipullista vuorokaudessa
2. Noin 10 - 20 savuketta, sikaria tai piipullista vuorokaudessa
3. Alle 10 savuketta, sikaria tai piipullista vuorokaudessa
4. Satunnaisesti

3) Ennen sairaalahoitoanne, montako vuotta yhteensä elämänne aikana olitte tupakoinut päivittäin?

Olin tupakoinut päivittäin noin _____vuotta.

4) Minkä ikäisenä aloititte päivittäisen tupakoinnin?

1. Alle 15-vuotiaana
2. 15 - 19-vuotiaana
3. 20 - 24-vuotiaana
4. 25-vuotiaana tai myöhemmin

5) Ennen sairaalahoitoanne, tupakoiko joku perheenjäsenistänne asuntonne sisätiloissa?

1. Ei kukaan
2. Kyllä, vain minä itse
3. Kyllä, joku muu perheenjäsen kuin minä
4. Kyllä, sekä minä että joku muu perheenjäsen

6) Entä jouduitteko silloin oleskelemaan työpaikallanne tupakansavuisissa tiloissa?

1. En koskaan
2. Kyllä, silloin tällöin
3. Kyllä, päivittäin tai lähes päivittäin
4. En ollut työelämässä

7) Tähän voitte vielä halutessanne tarkentaa tietoja tupakoinnistanne tai altistumisestanne tupakansavulle ennen sairastumistanne myyräkuumeeseen

_____

_____

_____

_____

8) Pituus ja painotiedot

Pituus_____ cm

Paino _____ kg

Paino ennen myyräkuumeeseen sairastumista (jos muistatte)_____kg

9) Mikä oli ammattinne, kun sairastuitte myyräkuumeeseen?

_____

Lämmin kiitos vaivannäöstänne!

ORIGINAL PUBLICATIONS

PUBLICATION I

Smoking is associated with aggravated kidney injury in Puumala hantavirus-induced haemorrhagic fever with renal syndrome

Tervo L, Mäkelä S, Syrjänen J, Huttunen R, Rimpelä A, Huhtala H, Vapalahti O, Vaheri A, Mustonen J

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Original Articles

Smoking is associated with aggravated kidney injury in Puumala hantavirus-induced haemorrhagic fever with renal syndrome

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ABSTRACT

Background. Previous studies indicate that smoking affects the outcome of some infections and is a risk factor for Puumala virus (PUUV) infection. The aim of this study was to assess the effect of smoking on the clinical severity of PUUV infection and the prevalence of smoking in patients with PUUV infection.

Methods. A questionnaire on smoking habits was sent to 494 patients in 2012, who had been treated in Tampere University Hospital, Finland, for serologically confirmed PUUV infection during years 1982–2012.

Results. Of all patients, 357 (72%) participated. Maximum plasma creatinine level measured during acute illness was significantly higher in current smokers than in non-smokers (median: 273 versus 184 μmol/L, $P < 0.001$). Current smokers had a higher maximum blood leucocyte count than non-smokers (median: 10.8 versus 8.9×10^9/L, $P < 0.001$) and they were younger than non-smokers (38 versus 45 years, $P < 0.001$). There were no differences between current smokers and non-smokers in the other variables reflecting the severity of PUUV infection. Altogether 51% were current smokers at the time of onset of the illness, 57% of males and 36% of females. During these years in Finland, smoking among males in the same aged population has decreased from 33 to 22% and among females, smoking has varied between 14 and 20%.

Conclusions. Smoking is common in patients with PUUV infection. Current smokers suffer from more severe acute kidney injury (AKI) and they have higher leucocyte count than non-smokers in PUUV infection. Smoking cessation decreases the risk of severe AKI to the same level as observed in never-smokers.

Keywords: acute kidney injury, acute tubulointerstitial nephritis, hantavirus infection, Puumala virus, smoking

INTRODUCTION

Puumala virus (PUUV) is a member of *Hantavirus* genus and it is carried by the bank vole (*Myodes glareolus*) [1]. Transmission of PUUV to humans occurs by inhalation of aerosols from infectious rodent excreta [1]. Hantaviruses have been recognized to cause two kinds of clinical syndromes in humans, haemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas [2]. However, increasing evidence suggests HFRS and HCPS to be the same disease, proposed to be called 'hantavirus disease' or 'hantavirus fever' [3–5]. Hantaviruses causing HFRS include PUUV, Dobrava, Hantaan and Seoul viruses [6]. PUUV-induced nephropathia epidemica, a mild form of HFRS, is common in Fennoscandia (Finland and Scandinavia) [2]. In Finland, ∼1000–3000 serological PUUV infection diagnoses are made annually and the seroprevalence in the population is 5% [7]. Large registry data from Finland have shown that 62% of cases are males, and the highest incidence is observed in the age group of 34–64 years [8].

The clinical course of PUUV infection varies from asymptomatic to fatal; the overall case fatality rate is low, ranging

from 0.08% [8] to 0.4% [9]. Host genetics influence the clinical picture [10–12]. The most typical symptoms in PUUV infection are high fever, headache, nausea, abdominal pain and lumbalgiae [6, 13]. Haemorrhages are rare. Typical laboratory findings are thrombocytopenia, leukocytosis, anaemia and elevated plasma C-reactive protein (CRP) [6, 13]. Renal involvement includes transient, sometimes massive proteinuria, microscopic haematuria and acute kidney injury (AKI), which is followed by polyuria and spontaneous recovery. Up to 6% of hospitalized patients need transient haemodialysis treatment [6, 13]. The characteristic renal histopathological finding is acute tubulointerstitial nephritis [1].

Smoking is known to affect the severity of some bacterial and viral infections, such as pneumonia, influenza and tuberculosis [14, 15]. In the respiratory tract, smoking leads to structural and functional changes [14], which may alter both susceptibility to and the course of infection. In addition to smoking, other factors, such as age, gender, body mass index (BMI) and comorbid diseases, affect the incidence and severity of some infectious diseases [14, 16, 17].

Epidemiological studies have concluded that smoking is an independent risk factor for the development of proteinuria, progression of diabetic nephropathy, progression of chronic kidney disease (CKD) and graft failure after kidney transplantation [18, 19]. However, the role of cigarette smoking in development of AKI has been less studied. This may be an important and underestimated entity, probably commonly masked by other risk factors of AKI, such as advanced age, obesity and comorbid conditions. Recently, a novel mechanism by which cigarette smoking may be involved in the development of AKI has been reported [20]. Chronic nicotine exposure may increase production of reactive oxygen species (ROS) and mitochondrial depolarization resulting in an injury in proximal tubule cells [20].

There are two previous reports of smoking as a risk factor to PUUV infection. Considerable odds ratios (ORs) of 9.1 and 3.6 were obtained in case–control studies performed in Belgium and Finland, respectively [21, 22]. However, there are no previous data on the effect of smoking on clinical course of PUUV infection or other hantavirus infections. The aim of this study was to investigate whether smoking affects the severity of the PUUV infection and to define the prevalence of smoking among Finnish patients with PUUV.

MATERIALS AND METHODS

Subjects

The study was carried out in Tampere University Hospital, Finland. All patients gave informed consent to participate and the study protocol was approved by the Ethics Committee of Tampere University Hospital. The study population consisted of 569 PUUV patients who had participated in our previous clinical studies [10–12, 23]. Patients had been treated at Tampere University Hospital with serologically confirmed PUUV infection [7, 24–26] during the years 1982–2012. Data on smoking habits were collected by a questionnaire sent to 494 patients from January to March 2012. Addresses could not be found in 28 patients, 2 were living abroad and 45 were deceased at the time of the study. Out of all patients, 357 (72%) participated in the study. The design of the study is described in Figure 1.

The mean age of participants was 41.3 (SD 12.2) years at the onset of disease and 257 (72%) of them were males. Forty patients had at least one of the following diseases before PUUV infection: hypertension, coronary artery disease, hypercholesterolaemia or diabetes. Hypertension was seen in 24 patients, coronary artery disease in 8 patients, diabetes in 7 patients and hypercholesterolaemia in 7 patients. Previous asthma had been diagnosed in eight patients and hypothyreosis in six patients. In addition, there were some separate chronic diseases in 24 patients. Otherwise the patients had been relatively healthy and 79% of them did not have any chronic diseases prior to PUUV infection. None of the patients had any known CKD prior to acute illness. Patients had not received non-steroidal anti-inflammatory drugs during their hospital stay.

Laboratory markers and clinical variables

Plasma creatinine and CRP concentrations, blood leucocyte and thrombocyte counts and blood haematocrit were determined by standard methods. The highest and lowest values of the various variables measured during hospitalization for each patient were designated as the maximum and minimum values, respectively. The clinical variables, such as length of hospitalization, systolic and diastolic blood pressure and change in weight (reflecting fluid retention during the oliguric phase) during acute illness, were obtained. Severe AKI was defined by plasma creatinine level equal to or more than 353.6 μmol/L or need of dialysis. This definition of severe AKI meets the criteria of KDIGO Clinical Practice Guidelines for AKI Stage 3 [27].

Questionnaire

The data of smoking history were collected by a questionnaire. The questionnaire contained six questions concerning smoking habits before and at the onset of PUUV infection. Previous and current smoking status was asked as well as the

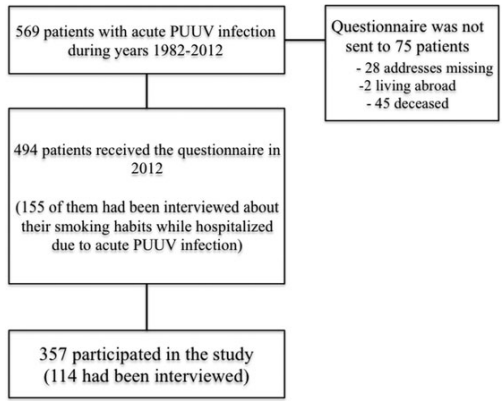

FIGURE 1: Design of the study.

Table 1. Maximum plasma creatinine concentration and blood leucocyte count in current smokers and in non-smokers (ex-smokers and never-smokers)

	Current smokers		*n*	Non-smokers		*n*	P
	Median	Range		Median	Range		
All patients			183			174	
Plasma creatinine max. (μmol/L)	273	51–1645		184	52–1537		<0.001
Blood leucocyte count max ($\times 10^9$/L)	10.8	4.1–44.7		8.9	3.8–50.3		<0.001
Males			147			110	
Plasma creatinine max. (μmol/L)	292	71–1645		191	67–1537		0.005
Blood leucocyte count max ($\times 10^9$/L)	11.0	5.4–44.7		9.2	3.8–50.3		0.005
Females			36			64	
Plasma creatinine max (μmol/L)	227	51–1156		175	52–959		0.529
Blood leucocyte count max ($\times 10^9$/L)	9.7	4.1–43.3		8.6	4.2–31.2		0.030

Differences between genders are also shown. Values are expressed as median and ranges.

lifelong exposure to cigarette smoke at home or at work (passive smoking). The amount of daily smoking was divided into four groups: occasionally, under 10 cigarettes/cigars/pipes per day, 10–20 cigarettes/cigars/pipes per day and over 20 cigarettes/cigars/pipes per day.

According to the questionnaire, patients were divided into three groups: current smokers, ex-smokers and never-smokers. Current smokers smoked at the time of the onset the illness. Ex-smokers had quit smoking at some stage before acute PUUV infection, and never-smokers reported that they had never smoked. To evaluate the effect of current smoking on the severity of acute PUUV infection, the patients were divided into two groups: current smokers and non-smokers. The group of non-smokers included both never-smokers and ex-smokers.

Occupational status of the patients was considered as a potential confounder when studying the relationship between smoking and PUUV. The patients were divided into three groups in accordance with Statistics Finland's occupational categories (http://www.tilastokeskus.fi). More than half of the patients were blue-collar workers (54%), one-fourth were upper white-collar workers (24%) and the rest were lower white-collar workers (22%). Students and pensioned ($n = 35$) were excluded from the analysis, because there was no knowledge of their future or previous occupation.

The validity of the smoking information

The research nurse interviewed the patients during their hospital stay during the years 2005–12. The current smoking status at that time was asked. One hundred fifty-five (31%) of 494 patients to whom the questionnaire was sent had been interviewed (see Figure 1). According to this interview, smoking habits were known in 114 patients, who responded to the questionnaire, and in 41 of non-respondents. These interview data were compared with the data obtained from the questionnaire to test the validity of the questionnaire and the memory of respondents. There were differences in 2 (1.8%) out of 114 answers only between the questionnaire and the interview. Therefore, we consider the questionnaire data on smoking valid.

Analysis of non-respondents

Between respondents and non-respondents, there were no differences between gender distribution, BMI or clinical severity of PUUV infection (maximum creatinine level, maximum leucocyte count, minimum thrombocyte count, change of weight during acute illness or length of hospital stay). In this respect, respondents represented well the entire study population. Non-respondents were slightly younger than respondents at the time of the infection (mean age: 37 versus 41, $P < 0.001$). Based on the interview of the research nurse, non-respondents were more likely to be current smokers than respondents (73 versus 49%, $P = 0.018$).

Statistical analysis

The SPSS (version 20, IBM, Chicago, IL) statistical software package was used for statistical analyses. Data are presented as medians and ranges for continuous variables and numbers and percentages for categorical variables. Comparisons between the groups were made with Mann–Whitney *U*-test for continuous variables, while Pearson chi-square test or Fisher's exact test was used for categorical data. Correlations were calculated by the Spearman's rank correlation test. Logistic regression analysis was carried out to identify factors determining the severity of AKI. ORs were expressed with their 95% confidence intervals (CI). All tests were two sided and a P-value < 0.05 was considered as statistically significant.

RESULTS

Altogether 183 out of 357 (51%) patients were current smokers at the time of PUUV infection. Of non-smokers, 56 (16%) were ex-smokers and 118 (33%) had never smoked. Smoking was more prevalent in men than in women: 57% of men (147/257) and 36% of women (36/100) were current smokers ($P < 0.001$). Current smokers were younger than non-smokers (38 versus 45 years, $P < 0.001$), and they had fewer chronic diseases than the non-smokers (13 versus 29%, $P < 0.001$). Non-smokers had cardiovascular diseases and/or diabetes more often than current smokers (16 versus 6%, $P = 0.001$). Smoking was most prevalent among blue-collar workers, of which 62% were current smokers, while 53 and 38% of lower white-collar and upper white-collar workers were current smokers, respectively ($P = 0.002$).

As shown in Table 1, the median maximum plasma creatinine level was significantly higher in current smokers than in

non-smokers (273 versus 184 μmol/L, P < 0.001). This difference was statistically significant in men only. Seventy-five of 183 (41%) current smokers developed severe AKI (plasma creatinine level equal to or higher than 353.6 μmol/L) compared with 46 out of 174 (26%) of non-smokers (P = 0.005).

In logistic regression analysis with severe AKI as a dependent variable and smoking status (current smoker versus non-smoker), age, gender and occupational category (three categories) as independent variables, the only significant factor determining severe AKI turned out to be smoking status (OR: 1.8; 95% CI: 1.1–3.0). Previous hypertension, coronary artery disease, hypercholesterolaemia or diabetes did not influence the risk of severe AKI. There were no differences in maximum plasma creatinine level in patients with or without such comorbidities (172 versus 226 μmol/L, P = 0.360).

The amount of smoking did not affect the severity of AKI. Maximum creatinine level did not differ between heavy smokers, who smoked >20 cigarettes per day, and those who smoked less, i.e. 1–19 cigarettes per day (275 versus 271 μmol/L, P = 0.470). There were only 14 such never-smokers, who had been exposed to cigarette smoke at work or at home (passive smokers). There was no difference in median maximum creatinine level in 104 never-smokers, who had never been exposed to cigarette smoke compared with 14 passive smokers (223 versus 206 μmol/L, P = 0.973).

To evaluate the effect of previous smoking on the severity of acute PUUV-induced AKI, we compared a group of never-smokers with those who had smoked previously but stopped smoking at some stage before PUUV infection (ex-smokers). There were no significant differences in maximum plasma creatinine level between these two subgroups (163 versus 197 μmol/L, P = 0.563).

As shown in Table 1, median maximum blood leucocyte count was significantly higher in current smokers than in non-smokers. This difference was seen in both genders. Leucocyte count did not differ between heavy smokers (>20 cigarettes per day) and those who smoked 1–19 cigarettes per day (10.6 versus 10.8×10^9/L, P = 0.874). There was no difference in leucocyte count between ex-smokers and never-smokers (8.8 versus 9.0×10^9/L, P = 0.314). A correlation between maximum creatinine level and maximum leucocyte count was found ($r = 0.432$, P < 0.001).

There were no differences between current smokers and non-smokers in other markers of disease severity, i.e. minimum or maximum haematocrit level, minimum thrombocyte level, duration of hospital stay or change of weight during the acute illness (data not shown). Plasma CRP level, the lowest or highest systolic or diastolic blood pressure measured during hospital care or BMI did not differ between smokers or non-smokers (data not shown).

DISCUSSION

The present large cohort study showed that current smokers had more severe PUUV-induced AKI than non-smokers. Those who had quit smoking at some stage before acute infection did not have such an increased risk. The number of daily smoked cigarettes did not influence the severity of AKI. Current smokers also had significantly higher maximum blood leucocyte count than non-smokers. More than half of the patients were current smokers at the time of disease onset, which is clearly more than in the average population in Finland. This finding confirms previous observations of smoking as a risk factor for PUUV infection [21, 22].

There are studies indicating that smoking may affect the outcome of bacterial and viral infections, such as pneumococcal pneumonia and influenza [14, 15]. A recent study by Bello *et al.* [15] showed that active smoking increases the risk of death from pneumococcal pneumonia independently of age and comorbid conditions. Smoking has also been found to be a risk factor for fatal outcome of human avian influenza A (H7N9) [28]. To our knowledge, no previous studies have been published on smoking and the severity of PUUV or other hantaviral infections.

In the present study, current smokers had higher maximum plasma creatinine level than non-smokers. This difference was statistically significant in males only. However, a similar trend was seen in females. The number of female smokers was small ($n = 36$), which might have affected the results. Interestingly, ex-smokers did not have more severe AKI than never-smokers, reflecting the reversibility of adverse effects of cigarette smoking after cessation.

The data of smoking history were collected by a questionnaire. We recognize the limitations of this kind of study design. There are, however, studies indicating that the validity of self-reported smoking is high and smokers very rarely claim to be non-smokers when self-reporting their smoking habits [29, 30]. Further, there was no inconsistency between the results of the questionnaire and the interviewed data of smoking.

Advanced age is a known risk factor for AKI. In the present study, current smokers were younger than non-smokers. The odds for developing severe AKI among the current smokers was 1.8 compared with the non-smokers. According to logistic regression analysis, when smoking status, age, gender and occupational category were chosen as independent variables, the only significant factor determining severe AKI turned out to be smoking status. Pre-existing CKD is a well-known risk factor for AKI. None of our patients had any known CKD prior to PUUV infection. In this previously relatively healthy population, vascular comorbidities or diabetes diagnosed prior to PUUV infection did not predispose to severe AKI, either.

Smokers had less chronic diseases than non-smokers. Smoking is known to predispose to cardiovascular diseases, but the effects of smoking are not usually seen before the age of 40 years. In the present study, smokers were younger than non-smokers (38 versus 45 years), which may explain the unexpected difference in the prevalence of chronic diseases between smokers and non-smokers.

In the present study, severe AKI was defined according to recent criteria of KDIGO Clinical Practice Guidelines for AKI (plasma creatinine level equal to or more than 353.6 μmol/L, or need of dialysis therapy). We did not use estimated glomerular filtration rate equations in our analysis, since the Modification of Diet in Renal Disease equation and other formulas have been developed in patients with stable CKD, and are inappropriate

for use in AKI [31]. We are aware that all creatinine-based definitions of AKI can be misleading in patients whose creatinine kinetics and volume of distribution are variable [31].

The knowledge of association between smoking and development of AKI is limited. The suggested mechanisms of smoking-induced renal damage include non-haemodynamic and haemodynamic factors [18]. Smoking is known to increase blood pressure and heart rate, mainly because of the effects of nicotine [32]. Smoking also activates the renin–angiotensin system, which is thought to be one element in the pathophysiology of smoking-induced renal damage in CKD [33]. In the present study, there were no differences in acute-phase blood pressure levels between current smokers and non-smokers. That might indicate that smoking-related non-haemodynamic rather than haemodynamic mechanisms are involved in the development of PUUV-induced AKI.

Epidemiological reports have shown that smoking exacerbates the progression of CKD [19, 34]. Potential non-haemodynamic mechanisms affecting the progression of CKD are heavy metals, hypoxia, prothrombotic factors, oxidative stress, proinflammatory cytokines and the activation of nicotine acetylcholine receptors [18]. The knowledge about those mechanisms leading to AKI is limited. Smoking elevates oxidative stress/production of ROS in various organs including kidneys. The mitochondria are known to be affected by cigarette smoke. Arany *et al.* [20] have recently shown in a mouse model that chronic nicotine exposure increases protein p66shc expression resulting in mitochondrial ROS production and depolarization of mitochondria, consequently leading to injury in cultured proximal tubule cells.

Smoking can also lead to endothelial dysfunction [32]. Hantaviruses replicate in the endothelial cells without visible cytopathic effects [1, 6]. Hantavirus infection of endothelial cells leads to dysfunction of the normal barrier function of the endothelium causing capillary leakage [1, 6]. In the present study, patients who were current smokers were at increased risk to get severe AKI, but the amount of daily smoked cigarettes or history of ex-smoking did not influence the severity. There might, thus, be differences in the function of endothelium just at the time of PUUV infection between smokers and non-smokers leading to differences in the outcome.

A characteristic histopathological renal finding in acute PUUV infection is acute tubulointerstitial nephritis without specific glomerular changes [1]. Morphological abnormalities have been observed in the proximal tubular epithelium after exposure to chronic cigarette smoking [35] and low-grade damage of proximal tubules has also been found among smokers in the general population [35]. These pre-existing morphologic alterations in tubular cells among smokers might influence the severity of PUUV-induced acute tubulointerstitial nephritis. It is also theoretically possible that smoking may influence the infiltration of macrophages and T cells into interstitium during acute interstitial nephritis.

The maximum blood leucocyte count was significantly higher among current smokers compared with non-smokers. Chronic cigarette smoking produces 20–25% increase in the number of peripheral blood leucocytes [36]. The increase may be due to generalized stimulation of the bone marrow with the higher turnover of all leucocyte lines [36]. Leukocytosis is considered to be one marker of PUUV infection severity [37]. Also in this study, there was a correlation between the maximum leucocyte count and creatinine level.

In the Belgian pioneer study by Van Loock *et al.* [21], smoking was found to be a risk factor for PUUV infection with a high OR of 9.1. This finding was confirmed in the previous Finnish study [22] as well as in the present study. A Belgian study also showed that the amount of daily smoked cigarettes influenced the risk of getting PUUV infection [21].

In our study, more than half of the patients were current smokers at the time of onset of the illness. Among men 57% and among women 37% smoked at that time. During the years 1982–2012, smoking in Finland among males decreased from 33 to 22% and among females, it has varied between 14 and 20% (www.thl.fi). A similar trend was found among the study populations, but during every decade, the study populations smoked clearly more than the average population in Finland (Figure 2).

Active smoking is known to increase the risk of developing influenza, community-acquired pneumonia and invasive pneumococcal disease, all transmitted through inhalation [14]. Also transmission of PUUV to humans occurs through inhalation of aerosols of infectious rodent excreta. It has been hypothesized that the condition of respiratory tract influences whether the infectious aerosol enters the alveoli and stays there long enough to cause the infection [22]. Smoking is known to lead to structural and functional changes in the respiratory tract [14]. In addition, smoking has effects on the local and systemic immune system [14]. These changes may alter both susceptibility to and the course of infection. One possible mechanism explaining smoking as a risk factor for severe PUUV infection could be higher local viral load due to effects of smoking to the respiratory tract. In fact, an early high viral load is thought to predict unfavourable outcome both in HCPS and in PUUV-induced HFRS [1].

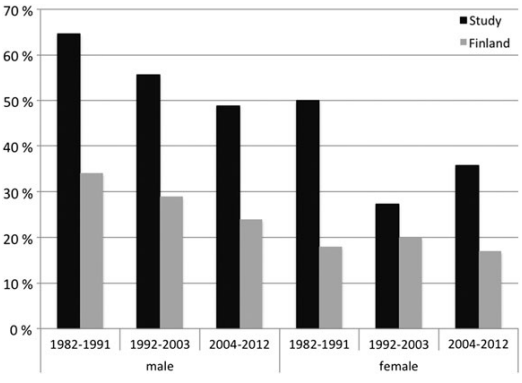

FIGURE 2: Percentage of daily smokers in 357 patients with acute PUUV infection (dark columns) and in the 15- to 64-year-old Finnish population (light columns) during three time periods in both genders (http://www.julkari.fi/bitstream/handle/10024/110537/URN_ISBN_978-952-245-931-2.pdf?sequence=1). The bar represents the average value within the presented time period.

In conclusion, current smokers are at clearly increased risk to suffer severe PUUV-induced AKI. The pathogenic mechanisms need further studies. Smoking cessation decreases the risk of severe AKI to the same level as never-smokers. These findings could be useful for clinicians to encourage the patients for smoking cessation.

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CONFLICT OF INTEREST STATEMENT

None declared. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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PUBLICATION II

Alcohol consumption and its influence on the clinical picture of Puumala hantavirus infection

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Article

Alcohol Consumption and Its Influence on the Clinical Picture of Puumala Hantavirus Infection

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Abstract: Puumala hantavirus (PUUV) causes hemorrhagic fever with renal syndrome. Characteristic clinical findings include acute kidney injury (AKI), thrombocytopenia, and capillary leakage. Smoking increases the risk of severe AKI, but it is not known whether alcohol consumption predisposes patients to a more severe infection. Liver and pancreatic enzymes, as well as biomarkers of alcohol consumption (gamma-glutamyl transferase, GGT; carbohydrate-deficient transferrin, CDT; GGT-CDT combination; and ethyl glucuronide, EtG), were measured from 66 patients with acute PUUV infection during hospitalization and at the convalescence phase. Alcohol consumption was present in 41% of the study population, 15% showing signs of heavy drinking. Alcohol use did not affect the severity of PUUV induced AKI nor the overall clinical picture of the infection. Liver enzyme levels (GGT or alanine aminotransferase, ALT) were elevated in 64% of the patients, but the levels did not associate with the markers reflecting the severity of the disease. Serum amylase activities at the convalescent stage were higher than those at the acute phase ($p < 0.001$). No cases with acute pancreatitis were found. In conclusion, our findings indicate that alcohol consumption does not seem to affect the clinical course of an acute PUUV infection.

Keywords: Puumala hantavirus; hemorrhagic fever with renal syndrome; alcohol drinking; ethanol; liver enzymes; pancreatitis; acute kidney injury

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1. Introduction

Nephropathia epidemica (NE) is a mild form of hemorrhagic fever with renal syndrome (HFRS). It is caused by Puumala hantavirus (PUUV), found in Europe and Russia [1]. The PUUV infection is carried by the bank vole (*Myodes glareolus*), and the infection is transmitted to humans by inhaling the aerosols of infected rodent excreta [2]. Other hantaviruses causing HFRS are Hantaan, Dobrava, and Seoul viruses [2]. The fatality rate of NE is low, ranging from 0.08% up to 0.4% [3,4]. The disease burden in Finland is considerable with the incidence of diagnosed PUUV infections of 31 to 39 cases per 100,000 inhabitants [5]. According to a recent nationwide study, a seroprevalence of 12.5% in the Finnish population was found [6].

The clinical picture of PUUV infection varies. Typical symptoms are fever, headache, nausea, abdominal pain, and backache [2,7]. The most typical laboratory findings are low platelet count, leukocytosis, anemia, and elevated plasma C-reactive protein (CRP) and creatinine levels, as well as hematuria and proteinuria [8]. A PUUV infection can be divided into five distinct phases: febrile, hypotensive, oliguric, polyuric, and convalescent

phases [8]. Renal involvement includes the oliguric phase with acute kidney injury (AKI), followed by polyuria and spontaneous recovery. A small percentage of patients need transient hemodialysis treatment [9]. Host genes have an influence on the outcome of PUUV infection [10–12]. Several biomarkers can predict the severity of the disease [8,13,14]. Recently, albuminuria, hematuria, and glucosuria, as well as hyperglycemia have been shown to predict the severity of PUUV infection [15–17].

Raised serum aminotransferases (ALT, AST) are reported as typical laboratory findings in HFRS, with a prevalence of 41–85% [18–21]. In a Swedish study, 60% of patients exceeded the upper normal limits [19]. The association of aminotransferase levels with the clinical course or symptoms in PUUV infection has, however, remained unknown.

The results concerning pancreatic involvement in HFRS have been contradictory in previous studies. There are reports of a small series of HFRS accompanied by acute pancreatitis [22–28]. In these studies, different hantaviruses are presented. To our knowledge, there is only one case report of PUUV induced acute pancreatitis [29]. In a German study of 166 patients with confirmed PUUV infection, serum lipase levels were elevated in 15% of patients at the acute phase of the disease [30]. Still, abdominal ultrasound or computer tomography (CT) scan showed no signs of acute pancreatitis. Patients with elevated lipase levels did not have abdominal pain more often than those with normal lipase levels, whereas they had a higher serum creatinine concentration, reflecting the severity of AKI [30].

In our previous study, we showed that current smoking is associated with severe AKI in PUUV infection [31]. Several lines of evidence have also indicated a significant association between alcohol consumption and smoking [32–34]. Excessive alcohol drinking raises serum liver enzymes, and it increases the risk of acute pancreatitis [35,36]. In addition to smoking, alcohol affects the host's immune defense. Alcohol use is known to predispose to various infections, such as bacterial pneumonia and systemic bacterial infections [37,38]. In animal models, alcohol exposure also predisposes to viral infections, such as respiratory syncytial virus pneumonia [39]. Furthermore, heavy drinking worsens the clinical outcome of several infections such as pneumonia, tuberculosis, and viral hepatitis [40]. However, there is no data on the possible association between alcohol consumption and the clinical outcome of PUUV infection or other hantavirus infections.

Gamma-glutamyltransferase, GGT, is an enzyme of the liver that has a 70–73% sensitivity to detect heavy alcohol consumption [41]. Another commonly used marker of alcohol consumption is carbohydrate-deficient transferrin, CDT, which has high specificity, but relatively low sensitivity [42]. Using a combination marker of GGT and CDT, formulated with the mathematical equation, the specificity and the sensitivity can be improved [43]. Another biochemical marker of alcohol consumption is ethyl glucuronide (EtG). It is a specific metabolite of ethanol, which can be measured from bodily fluids for several days after cessation of ethanol intake [43].

This study aimed to evaluate whether alcohol drinking affects the clinical outcome of PUUV infection. We also aimed to evaluate the changes in liver and pancreatic enzymes during acute PUUV infection and to assess their associations with patients' symptoms and other clinical and laboratory findings.

2. Materials and Methods

2.1. Subjects and Study Protocol

All patients were treated at Tampere University Hospital between January 2005 and November 2014 with serologically confirmed NE. The study cohort consisted originally of 86 consecutive patients of which 66 had control-phase serum samples available, and they were finally included in the study. All patients gave informed consent to participate, and the study protocol was approved by the Ethics Committee of Tampere University Hospital (R04180, R09206, R11188). The samples were collected daily during hospital care from day one at the hospital until discharge or until day five, whichever came first. Clinical variables were obtained during the hospital stay and symptoms were recorded accurately.

Convalescent phase samples of patients were obtained on median 24 (range 17–76) days after the onset of fever.

2.2. Laboratory Markers and Clinical Variables

Acute PUUV infection was serologically confirmed in all patients [44]. Blood leukocyte and platelet count, hematocrit, and plasma CRP concentration were determined by standard clinical chemical methods at the Laboratory Centre of Pirkanmaa Hospital District (later named Fimlab Laboratories), Tampere, Finland. The concentrations of serum alanine aminotransferase (S-ALT), serum gamma-glutamyl transferase (S-GGT), serum and urine (U) creatinine (S/U-creatinine), serum cystatin C (S-Cys-C), serum carbohydrate-deficient transferrin (S-CDT), serum and urine amylase (S/U-AMYL), urine trypsinogen (U-Trypsin), and urine ethyl glucuronide (U-EtG) were measured by accredited methods at the Laboratory of Seinäjoki Central Hospital, Seinäjoki, Finland. To detect recent alcohol consumption before sampling, U-EtG values above a cut-off limit of 500 ng/mL were used [45]. To improve sensitivity and specificity to detect heavy alcohol drinking, a combination marker of GGT and CDT was used; and, GGT-CDT was counted using a mathematical equation of GGT-CDT = 0.8 × ln(GGT) + 1.3 × ln(CDT). The cut-off limits of 4.3 for men and 3.8 for women were used [46]. Convalescent phase values of GGT-CDT were used to avoid the confounder effect of acute infection which may raise liver enzymes. Both alcohol markers (GGT-CDT and U-EtG) were available from 66 patients at the convalescent phase. Original data is available as Supplementary Material.

The highest or the lowest values of the variables measured during the hospital stay were designated as the maximum or minimum values. The severity of PUUV infection was evaluated with several variables. Cystatin C concentrations and the maximum serum creatinine reflected the severity of AKI. The severity of overall infection was also evaluated with a maximum blood leukocyte count and a minimum platelet count. Weight change during the hospital stay reflected fluid retention and, thus, both a change in the capillary permeability and the severity of AKI, whereas the length of hospital stay reflected the overall severity of the illness. Severe AKI was defined by a creatinine level equal to or more than 353.6 μmol/L which meets the criteria of KDIGO Clinical Practice Guidelines for AKI Stage-3 [47].

The definition of acute pancreatitis consisted of the following criteria: upper abdominal pain and serum amylase elevation threefold above the upper limit of normal [35]. Urine amylase/creatinine and trypsin/creatinine ratios were calculated to eliminate the bias due to urine concentration alterations. The detection limit for U-trypsin was 1.56 ng/mL, and concentrations below the detection limit were regarded as 1.56 ng/mL in the analyses. Clinical variables such as weight, blood pressure, and length of hospital stay, as well as symptoms, were documented accurately during the hospital stay. Cigarette smokers were classified into current smokers and non-smokers (including never smokers and ex-smokers).

2.3. Definition of Alcohol Use

We classified patients into three different categories. Patients were classified as alcohol heavy drinkers if the combination marker GGT-CDT exceeded the cut-off limit at the control stage. Patients with a U-EtG level above the cut-off limit but with GGT-CDT below the cut-off limit were classified as light drinkers. Patients who had no elevation in either of these markers were designated as abstainers. Patients were also divided into two groups: alcohol drinkers (including heavy and light drinkers) and abstainers.

2.4. Statistical Analysis

Data analyses were performed using IBM SPSS Statistics for Windows, version 27.0 (IBM Corporation, Armonk, NY, USA). To describe the data, medians and ranges were given for skewed continuous variables and numbers and percentages for categorical variables. To compare quantitative variables, a Mann–Whitney U-test was used. Categorical data were analyzed by the Chi-square test or the Fisher exact test, as appropriate. Spearman's

rank correlation test was used to find associations between liver and pancreatic enzyme levels and variables reflecting disease severity. When comparing between two groups (acute and convalescence phase values) a Wilcoxon signed-rank test was used, and when comparing three groups, the Kruskal–Wallis test was used. The *p*-values were adjusted by the Bonferroni correction for multiple tests. A two-sided *p*-value of less than 0.05 was regarded as statistically significant.

3. Results

The main laboratory findings and clinical characteristics are presented in Table 1. The median age was 41 years (range 22–74) and 36 (55%) of the patients were males. One or more of the following comorbidities was found prior to the onset of the disease in 17 patients: hypertension (n = 7), asthma (n = 2), rheumatoid arthritis (n = 2), type 2 diabetes (n = 3), coronary artery disease (n = 2), atrial fibrillation (n = 2), celiac disease (n = 1), transient ischemic attack (n = 1), and gastritis (n = 1). None of the patients had chronic kidney disease. Thirty-six (55%) patients were current smokers at the time of the onset of the illness. The patients were admitted to the hospital at the median of four days after the onset of fever. The clinical picture was typical of PUUV infection in all patients.

Table 1. Clinical and laboratory characteristics of 66 patients with acute PUUV infection.

Variable	Median	Range
Clinical variable		
Age (years)	41	22–74
BMI (kg/m^2) (n = 57)	26	19–37
Change in body weight (kg)	2	0–11
Length of hospital stay (days)	6	2–16
Systolic blood pressure on admission (mmHg)	126	72–182
Diastolic blood pressure on admission (mmHg)	80	40–110
Laboratory value		
Creatinine max (μmol/L)	175	53–1148
Cystatin C max (mg/L)	1.9	0.8–6.5
CRP max (mg/L)	91	16–267
Haematocrit max	0.44	0.33–0.60
Leukocyte count max ($\times 10^9$/L)	10.5	4.2–45
Platelet count min ($\times 10^9$/L)	53	5–150

Abbreviations: Max, maximum value during hospital stay; Min, minimum value during hospital stay; BMI, body mass index; CRP, C-reactive protein.

Out of 66 patients, 43 (65%) had at least one gastrointestinal (GI) symptom at the time of admission to the hospital. Thirty-eight patients (58%) had nausea, 33 (50%) vomited, and 18 (27%) had abdominal pain. When considering the markers reflecting alcohol consumption, S-GGT was found to be over the cut-off limit in 35/66 (53%) of the patients and S-CDT in 2 (3%) patients at the acute stage of NE. Both GGT and CDT were significantly higher during the acute phase when compared with the convalescence phase as shown in Table 2. We also calculated the magnitude of change between acute and control stages of the values presented in Table 2, and we compared these between alcohol drinkers and abstainers. This comparison was also made between heavy drinkers, light drinkers, and abstainers. There were no differences between the two groups (data not shown).

Table 2. Comparison of acute-phase values and control values. Control samples were taken at the median of 24 (range 17–76) days after the onset of fever.

	Acute Phase (n = 66)		Convalescent Phase (n = 66)		
	Median	**Range**	**Median**	**Range**	***p* Value** [a]
ALT max (U/L)	35	14–400	27	5–126	0.003
GGT max (U/L)	52	16–549	44	5–168	0.007
Amyl max (U/L)	48	13–181	74	20–215	<0.001
Trypsinogen max (ng/L)	6.6	1.6–33.7	1.6	1.5–11.1	<0.001
Trypsin/crea max	1.1	0.1–14.9	0.2	0.1–3.5	<0.001
CDT max (%)	1.6	1.0–3.4	1.5	1.0–2.8	0.005
GGT-CDT max					
women	3.5	2.7–5.1	3.3	2.7–4.9	0.072
men	3.9	2.5–6.1	3.7	1.8–4.8	0.034

Abbreviations: Max, maximum value during hospital stay; ALT, serum alanine aminotransferase; GGT, serum gamma-glutamyltransferase; Amyl, serum amylase; Trypsin/crea, urine trypsinogen: creatinine ratio; CDT, serum carbohydrate-deficient transferrin; GGT-CDT, combination marker of gamma-glutamyltransferase and carbohydrate-deficient transferrin. [a] Comparison between acute and convalescence phase values; Wilcoxon signed-rank test was used.

To improve the sensitivity and the specificity to identify heavy drinking, we calculated the combination marker GGT-CDT. In the analyses, we used GGT-CDT of 66 patients at the convalescent phase to avoid the confounder effect of acute infection, which may raise S-GGT. This combination marker showed elevated levels in 5/36 (14%) males and 5/30 (17%) females. Only one patient had high urine EtG (2786 ng/mL) already at admission; while, at the convalescent phase, 20 out of 66 (30%) patients showed urine EtG concentration >500 ng/mL, suggesting recent alcohol consumption. Using either a U-EtG (showing recent alcohol consumption) or a GGT-CDT-combination marker (showing heavy alcohol use) exceeding the cut-off limit, we found 27/66 (41%) patients with biochemical evidence of alcohol consumption before the control visit: 15/36 (42%) males and 12/30 (40%) females.

The median of maximum serum creatinine and cystatin C concentration at the acute phase did not differ between alcohol drinkers as compared with abstainers: S-creatinine 118 μmol/L (range 53–1148) vs. 200 μmol/L (range 62–1139), $p = 0.208$ and S-cystatin C 1.7 mg/L (range 0.8–5.0) vs. 1.9 mg/L (range 0.8–6.5), $p = 0.270$, respectively. There were no differences in other variables reflecting disease severity or gastrointestinal symptoms at admission (data not shown). Alcohol drinkers had slightly higher GGT as compared with abstainers: 57 U/L (range 18–295) vs. 50 U/L (range 16–549), $p = 0.043$. Fifty-two percent (14/27) of alcohol drinkers were current smokers, whereas 56% (22/39) of patients with no alcohol use smoked ($p = 0.715$).

We also compared heavy alcohol drinkers, light drinkers, and abstainers. There were no differences in a length of hospital stay, change in body weight, blood pressure on admission, the lowest platelet count, or in the severity of AKI between these groups as shown in Table 3.

Table 3. Comparison of laboratory values (median of maximum or minimum value) and clinical symptoms among heavy alcohol drinkers, light drinkers, and abstainers.

Variable	Use of Alcohol			*p*-Value [a]
	Heavy Drinker (*n* = 10)	Light Drinker (*n* = 17)	Abstainer (*n* = 39)	
Clinical variable				
Change in body weight (kg)	1.6	3.5	1.8	0.447
Length of hospital stay (days)	5	8	6	0.153
Systolic blood pressure on admission (mmHg)	127	129	124	0.994
Diastolic blood pressure on admission (mmHg)	80	82	86	0.754
Laboratory value				
ALT max (U/L)	33	34	35	0.967
GGT max (U/L)	98	49	50	0.005
Amyl max (U/L)	32	47	49	0.089
Creatinine max (μmol/L)	115	172	200	0.418
Cystatin C max (mg/L)	1.6	1.7	1.9	0.520
CRP max (mg/L)	99	99	82	0.266
Haematocrit max	0.41	0.44	0.45	0.123
Leukocyte count max ($\times 10^9$/L)	11.7	10.7	10.1	0.692
Platelet count min ($\times 10^9$/L)	78	60	43	0.142
Symptom				
Abdominal pain	1/10 (10%)	4/17 (24%)	13/39 (33%)	0.309
Nausea	5/10 (50%)	11/17 (65%)	22/39 (56%)	0.737
Vomiting	4/10 (40%)	9/17 (53%)	20/39 (51%)	0.785

Abbreviations: Max, maximum value during hospital stay; Min, minimum value during hospital stay; ALT, serum alanine aminotransferase; GGT, serum gamma-glutamyltransferase; Amyl, serum amylase; CRP, plasma C-reactive protein; max, maximum value during hospital stay; min, minimum value during hospital stay. [a] Comparison between three groups; Kruskal–Wallis test was used. *P*-values have been adjusted by the Bonferroni correction for multiple tests.

Severe AKI (creatinine >353.6 μmol/L) was not more frequent among heavy drinkers (2/10, 20%) or light drinkers (4/17, 24%) as compared with abstainers (13/39, 33%), $p = 0.607$. Clinical symptoms did not differ either between heavy alcohol drinkers, light drinkers, or abstainers (Table 3). We also wanted to compare heavy alcohol drinkers to the rest of the patients (light drinkers and abstainers). There were no differences in variables reflecting the disease severity, such as the length of hospital stay or the minimum platelet count. In the same way as with other comparisons, neither did the severity of AKI nor clinical symptoms differ between heavy users and other users (data not shown).

Serum ALT exceeded the upper normal limit in 24/66 (36%) and S-GGT in 35/66 (53%) patients at the acute phase of the disease. Both enzymes were elevated in 17/66 (26%) of the patients and one or the other in 42/66 (64%) of the patients. As shown in Table 2, the maximum median ALT and GGT concentrations were significantly higher during the acute phase compared with the values at the control visit. Neither ALT nor GGT correlated with the severity of AKI, lowest platelet count, or any other variable reflecting the severity of the acute infection (data not shown).

The serum amylase concentration was below the normal limit (120 U/L) in almost all patients (62/66, 94%) at the acute phase. None of the patients had major increases in the amylase levels and, interestingly, serum amylase levels were found to be significantly lower during the acute phase when compared with the convalescent phase (Table 2). The highest trypsinogen value in this cohort being 33.7 ng/mL, U-Trypsin was not elevated in any of the patients. All patients had U-trypsin/creatinine ratios within the normal limits.

Patients presenting with vomiting had higher S-ALT than those who did not: 45 U/L (range 17–400) vs. 31 U/L (range 14–87), $p = 0.009$. Otherwise, S-ALT or S-GGT levels did not differ between patients with or without GI symptoms. Patients with abdominal pain on admission showed higher serum amylase activities: 66 U/L (range 33–134) vs. 40 U/L (range 13–181), $p = 0.006$; but, no one showed serum amylase elevation threefold the upper limit of normal. They also showed higher serum creatinine and cystatin C levels compared with those without abdominal pain: 350 μmol/L (range 76–1139) vs. 133 μmol/L (range 53–1148), $p = 0.020$ and 2.1 mg/L (1.2–6.5) vs. 1.6 mg/L (0.8–5.4), $p = 0.087$, respectively.

4. Discussion

In the present study, forty-one percent of the patients showed some biochemical signs of alcohol use at the control visit. However, alcohol drinking was not found to show a significant relationship with the clinical course of PUUV infection. Liver enzymes were also commonly elevated in these patients, but they did not associate with the clinical outcome or kidney dysfunction. There were no cases of acute pancreatitis, and serum amylase levels were actually lower in the acute phase of the infection when compared with the convalescent stage.

Patients with evidence of alcohol consumption did not have a more severe clinical course of the acute infection. An enzyme of the liver, GGT is known to reflect alcohol overconsumption with a sensitivity of 70–73% [41]. However, GGT may also rise due to other factors including obesity, smoking, drug use, or some infections, which hampers its use as an alcohol marker, especially in hospital settings. Another widely used marker of alcohol abuse is CDT, which predicts heavy consumption with relatively high specificity but low sensitivity [42]. Both S-GGT and S-CDT are markers of chronic alcohol abuse, and they normalize within two to three weeks after the cessation of alcohol consumption [42,43].

A combination marker of GGT and CDT is more sensitive and specific to detect heavy alcohol consumption than either of these markers alone [42]. The combination marker GGT-CDT reacts when regular ethanol consumption exceeds 40 g per day [43]. In this work, we used the convalescence phase GGT-CDT levels for assessing alcohol consumption because we wanted to exclude the possible confounding effect of GGT elevation due to acute infection. This combined marker indicated heavy alcohol drinking in 14% of males and 17% of females. According to a large Finnish survey, with 13 976 individuals in the national FINRISK-study, the prevalence of heavy alcohol drinking in the general population was 4.2% [48]. In that study, alcohol consumption was assessed with a questionnaire, which may underestimate the true consumption. Nevertheless, our study showed over three times more cases with biomarker-based evidence of heavy alcohol consumption among the present patient population.

Some previous studies have shown that alcohol worsens the clinical outcome in infectious diseases [40]. In this cohort, neither heavy nor light alcohol use was found to predispose to more severe PUUV infection. The severity of AKI did not differ in alcohol drinkers compared with abstainers and there was no difference in other variables reflecting disease severity either. Alcohol drinkers did not show more frequent abdominal pain, vomiting, or nausea on admission to the hospital.

Ethyl glucuronide (EtG) is a specific metabolite of ethanol, which can be measured from bodily fluids for several days after cessation of ethanol intake [45]. Urine EtG can rise after a single instance of use, and it does not necessarily point to chronic alcohol abuse. With the cut-off limit of 500 ng/mL, other sources of ethanol exposure, such as the use of alcohol-containing hand disinfection or alcohol-containing cosmetics, are excluded. In the present study, urine EtG was elevated only in one patient at the acute phase of NE, whereas at the recovery stage 30% of the patients showed a urine EtG concentration over 500 ng/mL. Patients were admitted to the hospital at the median of four days after the onset of the fever. Since the half-life of EtG is short, the acute-phase levels reflect only the consumption of alcohol during the febrile stage of NE. It seems obvious that at the acute phase most patients did not use alcohol; and, therefore, only one patient showed

signs of alcohol consumption for a few days before hospitalization. On the other hand, at the convalescent stage, alcohol consumption had probably returned to the usual levels of habitual alcohol intake. Using either urine EtG or GGT-CDT-combination marker as indicators of ethanol consumption, we found that forty-one percent of the patients had used alcohol before the control visit. These patients, however, did not differ from abstainers with respect to clinical symptoms or the course of the disease. Cigarette smoking was common, but there was no difference in smoking status between alcohol drinkers and abstainers.

Liver enzymes, either GGT or ALT, were elevated in 64% of the patients. There were only a few patients with liver enzymes exceeding three-fold the upper limit of normal. In clinical practice, it is often seen that liver enzymes rise slightly during the acute course of many infections and then normalize without any specific treatment. Raised aminotransferases are typically reported during the acute phase of PUUV infection, but the clinical relevance of this observation is not clear [18–21]. While PUUV has been detected in liver from the lymphocytes of the sinusoids [49], PUUV has not been detected from hepatocytes, to our knowledge. Therefore, PUUV may induce inflammation in the liver without infecting the hepatocytes. Libraty and colleagues have previously shown that patients with severe AKI did not have higher serum ALT levels during acute PUUV infection, when compared with those with non-severe AKI [50]. In the present study, there was neither correlation between serum creatinine nor cystatin C levels and the levels of liver enzymes. Serum aminotransferases did not correlate with other markers of disease severity either. While alcohol abuse is also known to raise S-ALT levels [36], in this study the individuals classified as alcohol drinkers did not have high S-ALT activities, indicating that the quantities of alcohol consumed were probably below the thresholds that would cause significant liver damage.

Serum amylase concentrations in the present material were found to be low in almost all patients and, interestingly, the lowest serum amylase activities occurred at the acute phase of the disease. Patients sought treatment at a median of four days after the onset of fever. Presumably, patients had been feeling sick and lost appetite before admission to the hospital, and we speculate that preceding low nutrient intake due to acute illness may have contributed to the lower amount of salivary and total amylase levels in the acute phase.

Acute pancreatitis is associated with many infections, as also supported by recent observations among patients with SARS-CoV-2-infection [51]. The definition of acute pancreatitis consists of two out of three of the following criteria: (1) upper abdominal pain, (2) serum amylase or lipase elevation threefold above the upper limit of normal, and (3) computed tomography/magnetic resonance imaging/ultrasound findings of pancreatitis [35]. In this study, the definition of acute pancreatitis consisted of the following criteria: upper abdominal pain and serum amylase elevation threefold above the upper limit of normal. None of the patients met these criteria. Urine trypsinogen gives 100% specificity for acute pancreatitis using the cut-off limit of 100 ng/mL [52]. The highest trypsinogen value in this cohort reached 33 ng/mL, also ruling out cases of acute pancreatitis. There are reports of Hantaan, Dobrava, and Seoul virus-induced HFRS accompanied with acute pancreatitis [22–28]. In Europe, one study of 166 PUUV infected patients did not show any cases of pancreatitis [30]. Based on our findings and previous finding of Kitterer and colleagues [30], it seems that HFRS caused by PUUV does not induce acute pancreatitis.

Sixty-five percent of the patients had some gastrointestinal symptoms at the time of admission to the hospital. Abdominal pain is a common symptom in hantavirus infections for which there is no pathophysiological explanation, so far. A German study found the PUUV nucleocapsid antigen in 62% analyzed intestinal biopsies [53] and in the appendix of one patient [54], reflecting a generalized PUUV infection. In our study, patients with abdominal pain on admission had higher serum amylase and creatinine concentrations than patients without such symptoms. Greater amylase in these patients may be caused by impaired renal function as amylase is eliminated through the kidneys.

There are some limitations in this study, which must be acknowledged. We did not include systematic questionnaire-based interviews of the history and current use of alcohol,

such as the Alcohol Use Disorders Identification Test (AUDIT). Another limitation is the absence of other biomarkers, such as phosphatidylethanol (PEth). While PEth is currently known as a highly specific and sensitive biomarker of recent alcohol consumption, fresh blood samples are required for the analysis of this metabolite from red cells that were not available for this study [55]. The GGT-CDT combination marker, however, lacks only little in sensitivity (94%) compared with PEth [55]. Future studies should also address the possibility of whether the use of other biomarkers, such as mean corpuscular volume (MCV), could provide additional value in detecting alcohol consumption and its relation to disease severity in PUUV infected patients [56,57]. The serum and urine samples were frozen before the analyses were made. Freezing might affect the concentrations of some enzymes, but the time of freezing did not differ between acute and control phase samples, and we therefore we find the comparison of these values reliable. The study size is relatively small, but it is sufficient to detect a strong correlation. Further studies with a larger population would be of interest.

In conclusion, in the present study population, forty-one percent of the patients showed biomarker-based signs of recent alcohol consumption at the control visit. Alcohol use did not, however, affect the clinical outcome of the infection, and alcohol users did not have a more severe AKI. There were no cases of acute pancreatitis caused by PUUV infection. Unexpectedly, the serum amylase concentration was significantly lower during the acute phase compared with the control visit, which warrants further studies in larger materials.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14030500/s1. Flie S1: Original data.

Author Contributions: Conceptualization, S.M., J.T.M. and L.T.; methodology, L.T., O.N., T.K.O., S.M. and J.T.M.; software, L.T., H.H., T.K.O. and S.M.; validation, S.M. and J.T.M.; formal analysis, L.T., H.H. and T.K.O.; investigation, L.T., O.N., S.M., J.M., T.K.O. and J.T.M.; resources, O.N., S.M. and J.T.M.; data curation, L.T. and S.M.; writing-original draft preparation, L.T.; writing-review and editing, L.T., O.N., T.K.O., S.M., J.M., H.H., I.P., J.S. and J.T.M.; visualization, L.T., T.K.O., H.H., S.M., I.P. and J.T.M.; supervision, S.M., J.S. and J.T.M.; project administration, S.M. and J.T.M.; funding acquisition, L.T., S.M., O.N. and J.T.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Tampere University Hospital (R04180, R09206, R11188).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Original data is available as Supplementary Material.

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PUBLICATION III

The presence of intraperitoneal, retroperitoneal and pleural fluid in acute Puumala hantavirus infection

Tervo L, Outinen T, Kiekara T, Tietäväinen J, Paakkala A, Pörsti I, Huhtala H, Mäkelä S, Mustonen J

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PUBLICATION IV

Plasma levels of soluble urokinase-type plasminogen activator receptor associate with the clinical severity of acute Puumala hantavirus infection

Outinen TK, Tervo L, Mäkelä S, Huttunen R, Mäenpää N, Huhtala H, Vaheri A, Mustonen J, Aittoniemi J

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PLOS ONE

Plasma Levels of Soluble Urokinase-Type Plasminogen Activator Receptor Associate with the Clinical Severity of Acute Puumala Hantavirus Infection

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Abstract

Objectives: Urokinase-type plasminogen activator receptor is a multifunctional glycoprotein, the expression of which is increased during inflammation. It is known to bind to β_3-integrins, which are elementary for the cellular entry of hantaviruses. Plasma soluble form of the receptor (suPAR) levels were evaluated as a predictor of severe Puumala hantavirus (PUUV) infection and as a possible factor involved in the pathogenesis of the disease.

Design: A single-centre prospective cohort study.

Subjects and Methods: Plasma suPAR levels were measured twice during the acute phase and once during the convalescence in 97 patients with serologically confirmed acute PUUV infection using a commercial enzyme-linked immunosorbent assay (ELISA).

Results: The plasma suPAR levels were significantly higher during the acute phase compared to the control values after the hospitalization (median 8.7 ng/ml, range 4.0–18.2 ng/ml *vs.* median 4.7 ng/ml, range 2.4–12.2 ng/ml, $P<0.001$). The maximum suPAR levels correlated with several variables reflecting the severity of the disease. There was a positive correlation with maximum leukocyte count ($r=0.475$, $p<0.001$), maximum plasma creatinine concentration ($r=0.378$, $p<0.001$), change in weight during the hospitalization ($r=0.406$, $p<0.001$) and the length of hospitalization ($r=0.325$, $p=0.001$), and an inverse correlation with minimum platelet count ($r=-0.325$, $p=0.001$) and minimum hematocrit ($r=-0.369$, $p<0.001$).

Conclusion: Plasma suPAR values are markedly increased during acute PUUV infection and associate with the severity of the disease. The overexpression of suPAR possibly activates β_3-integrin in PUUV infection, and thus might be involved in the pathogenesis of the disease.

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Introduction

Hantaviruses cause two clinical syndromes in humans, hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas [1,2]. Puumala hantavirus (PUUV), carried by the bank vole, causes a mild HFRS called nephropathia epidemica (NE) [1,2]. NE is prevalent in Finland, elsewhere in Scandinavia, European Russia, and many parts of Central-Western Europe [1,2]. In Europe, PUUV causes most HFRS cases, and in Finland, 1,000–3,000 serological PUUV infection diagnoses are made annually (http://www3.ktl.fi) [3].

The clinical severity of NE varies from subclinical disease to fatal cases. However, the mortality rate is low, about 0.1% [4]. Typical symptoms are sudden high fever, headache, abdominal pain, nausea, backache, and visual disturbances, while serious hemorrhagic manifestations are uncommon [5–8]. Signs of renal involvement are proteinuria, hematuria, and oliguria, the latter being followed by polyuria [5–8]. During the oliguric phase, about 5% of hospitalized patients need transiently hemodialysis treat-

ment [6–8]. Usual laboratory findings are leukocytosis, thrombocytopenia, anemia, elevation of plasma C-reactive protein (CRP) and creatinine levels, as well as proteinuria and hematuria [6,7]. Radiological pulmonary manifestations have been detected in about one-third of hospitalized patients [9,10].

The pathogenesis of NE is incompletely understood. An important feature in hantavirus infections is universally increased capillary permeability leading to vascular leakage, but the mechanisms behind this phenomenon are unclear [11–13]. The endothelium of the small vessels in various organs is the primary target of hantavirus infection and β_3-integrins mediate the cellular entry of pathogenic hantaviruses [14,15]. It has been suggested that immunological factors rather than direct cytotoxicity are essential in the pathogenesis, since no obvious damage to the endothelial cells is seen [11–13].

Several biomarkers have been shown to serve as indicators of the severity of PUUV infection. The plasma levels of interleukin (IL)-6, pentraxin-3 (PTX3), and indoleamine 2,3 dioxygenase (IDO), all elements of the innate immunity, as well as of cellular damage -reflecting cell-free DNA (cf-DNA) predict the outcome of PUUV infection [16–19]. So does the urine levels of the transcription factor necessary for the generation of type 2 T-cells, GATA-3 [20]. On the contrary, although plasma CRP is elevated in almost all of the patients with PUUV infection, high CRP level does not indicate a more severe disease [19].

Urokinase-type plasminogen activator receptor (uPAR) is a multifunctional glycoprotein, the expression of which is increased during infection and inflammation [21,22]. It is expressed on several different cell types, including monocytes, activated T-lymphocytes, macrophages, neutrophils, endothelial cells, and kidney podocytes [21,22]. uPAR interacts with several molecules mediating immune system signals and promotes the migration and adhesion of leukocytes by binding to β-integrins [21,22]. Cell-cell contact has been shown to enhance the release of the soluble form of the receptor (suPAR) by endothelial cells, indicating a regulatory role of suPAR in cell adhesion [23]. The levels of plasma suPAR are considered to represent the degree of immunoactivation [22]. Plasma suPAR has been found to be increased as well as predict disease severity in various conditions, e.g. autoimmune diseases, cancer, malaria, tuberculosis, sepsis, and human immunodeficiency virus (HIV) infection [22,24–30].

In the present study, our aim was to evaluate the association of suPAR with the severity of acute PUUV infection and also assess the possible role of suPAR in the pathogenesis of the disease.

Materials and Methods

Ethics statement

All subjects gave a written informed consent before participation and the study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Ethics Committee of Tampere University Hospital.

Patients

The study cohort consisted of 97 prospectively collected adult patients with acute NE. The diagnosis of acute PUUV infection was serologically confirmed in all cases [31]. The patients were treated at the Tampere University Hospital (Tampere, Finland) from November 2001 to February 2009. The median patient age was 41 (range 22–77) years, and 63 (65%) were males. Part of the patients (10–83 patients of the present cohort depending on the previous study) has participated also in our previous studies [16–20,32–35]. All the patients treated during November 2001 and February 2009 were included in the study, if there was plasma left for the analyses of suPAR concentration.

Study protocol

All 97 patients were examined during the acute phase of NE. A detailed past and current medical history was obtained, and a careful physical examination was performed. Blood samples were collected between 7:30–9:30 in the morning for two consecutive weekdays after hospitalization for the analysis of plasma suPAR. Blood samples for the determination of plasma cf-DNA, PTX3, IL-6, CRP, creatinine, and serum kynurenine (Kyn) and tryptophan (Trp) levels, as well as the blood cell counts were collected for up to five consecutive days. Other blood samples were taken according to the clinical needs of the patient.

The highest and the lowest values of the various variables measured during hospitalization for each patient were designated as the maximum and minimum values.

Eighty-four (87%) of the 97 patients were also studied at the out-patient clinic 1–4 weeks after the hospital period. The plasma samples taken at the out-patient clinic after the hospital treatment were regarded as control samples.

Methods

EDTA-treated plasma samples for suPAR determination were collected from patients during hospitalization and at the out-patient clinic and stored at $-70^{\circ}C$ until required for analysis. Plasma suPAR levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA) (suPARnostic® Standard kit;ViroGates A/S, Birkerød, Denmark) according to the manufacturer's instructions.

Plasma CRP and creatinine levels were analyzed using Cobas Integra analyzer (F. Hoffman-La Roche Ltd, Basel, Switzerland). Blood cell count was completed by hematological cell counters by Bayer. Plasma IL-6 concentrations were determined as previously described [36]. IDO level can be measured by determining the ratio of Kyn to Trp in serum [37] by reverse-phase high performance liquid chromatography (HPLC) as previously described [38]. The Kyn/Trp ratio was calculated by relating concentrations of Kyn to Trp. Plasma PTX3 determinations were performed by using a commercially available human pentraxin-3 immunoassay (Quantikine, R&D Systems, Inc., Minneapolis, MN), following the manufacturer's instructions. Plasma cf-DNA levels were determined as previously described [16]. Plasma IL-6, PTX3, and cf-DNA, as well as serum Kyn and Trp concentrations were measured afterwards from frozen samples.

Statistical Analyses

In order to describe the data, medians and ranges were given for continuous variables and numbers and percentages for categorical variables. Groups were compared using the Mann-Whitney *U*-test. Correlations were calculated by the Spearman's rank correlation test. Wilcoxon's test was used to compare two related samples. All tests were two-sided, and statistically significant *P*-values are given. All analyses were made with the SPSS (version 20) statistical software package (IBM, Chicago, IL).

Results

The clinical characteristics of the patients are shown in Table 1 and the laboratory variables measured during hospitalization in Table 2. None of the patients was in clinical shock at the time of admission and five patients (5%) needed hemodialysis treatment during the hospital stay. No deaths occurred.

Table 1. Clinical data of 97 patients with acute Puumala hantavirus infection.

	Median	Range
Age (years)	41	22–77
Duration of fever before hospital admission (days)	4	1–15
Total duration of fever (days)	7	2–19
Length of hospital stay (days)	6	2–15
Change in weight during hospitalization (kg)	2.1	0–12.0
Minimum urinary output (ml/day)	1400	50–5800

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The maximum plasma suPAR levels taken during acute NE were significantly elevated compared to the control values taken after the hospitalization period (median 8.7 ng/ml, range 4.0–18.2 ng/ml *vs.* median 4.7 ng/ml, range 2.4–12.2 ng/ml, $P<0.001$). The control values were taken median 22 (range 15–41) days after the onset of fever. Figure 1 shows the suPAR levels in relation to the duration of the disease, i.e. the onset of fever.

The maximum plasma suPAR levels correlated with several parameters reflecting the severity of acute PUUV infection (Table 3). There was a positive correlation of suPAR with the length of hospital stay and with the change in weight during hospitalization, which reflects fluid retention during the oliguric phase. Plasma suPAR also correlated positively with plasma creatinine, CRP, PTX3, IL-6, IDO, and cf-DNA levels, as well as with the blood leukocyte counts. An inverse correlation was detected between maximum plasma suPAR level and minimum urinary output as well as between maximum suPAR and minimum platelet levels. There was no correlation between maximum suPAR and age (Table 3).

The maximum suPAR level was higher in patients who required dialysis treatment during hospitalization compared to patients who managed without dialysis (Table 4). Patients who stayed longest (>6 days) at the hospital had also higher suPAR levels than patients with shorter hospitalizations. Furthermore, patients with maximum creatinine level >200 μmol/l or maximum blood leukocyte count $>10\times10^9$/l had higher suPAR levels compared to patients with lower creatinine or leukocyte values. Significant thrombocytopenia (platelet count $<50\times10^9$/l) associated also with higher suPAR levels. There was no difference in suPAR levels between men and women (Table 4).

Discussion

In the present study, plasma suPAR levels in a hantavirus infection are reported to our knowledge for the first time. The data presented here show that plasma suPAR levels are clearly elevated during acute PUUV infection. The suPAR values measured during the acute phase were markedly higher than the control values measured after the hospitalization and correspond with the levels previously reported in bacteremic patients [25]. Furthermore, the convalescence phase levels correspond to the levels previously measured in the general population [39]. The maximum plasma suPAR levels also correlated with several PUUV infection severity reflecting variables in the present study. suPAR correlated positively with the length of hospital stay, the change in body weight during hospitalization, plasma creatinine, and the leukocyte counts. An inverse correlation was detected by suPAR with minimum urinary output and minimum platelet levels.

Table 2. Laboratory data of 97 patients with acute Puumala hantavirus infection.

	Median	Range
suPAR max (ng/ml)	8.7	4.0–18.2
Creatinine max (μmol/l)	175	51–1499
Platelets min (10^9/l)	61	9–187
Hematocrit min	0.36	0.25–0.44
Leukocytes max (10^9/l)	10.4	3.9–31.2
CRP max (mg/l)	84.6	15.9–269.2
IL-6 max (pg/ml) (n = 29)	11.8	2.6–44.8
PTX3 max (ng/ml) (n = 42)	42.9	3.9–1085.5
IDO max (μmol/mmol) (n = 83)	202.2	47.7–3679.2
cf-DNA (μmol/ml) (n = 42)	1.35	1.04–3.29

Min = minimum, Max = maximum, suPAR = soluble urokinase-type plasminogen activator receptor, CRP = plasma C-reactive protein, IL-6 = plasma interleukin-6, PTX3 = plasma pentraxin-3, IDO = serum indoleamin 2,3-dioxygenase, cf-DNA = plasma cell-free DNA.

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There was also a positive correlation between suPAR and the biomarkers previously shown to predict the outcome of PUUV infection, PTX3, IL-6, IDO, and cf-DNA [16–19]. PTX3, IL-6, and IDO not only reflect the severity of PUUV induced NE, but also the activation of the immune system, and are partly expressed by the same type of cells as suPAR in response to inflammatory signals. Furthermore, cf-DNA reflects the degree of cellular damage. CRP, on the other hand, does not predict the outcome of PUUV infection [19]. In the present study, however, suPAR correlated also with CRP in PUUV-infected patients. This probably is explained by CRP reflecting the activation of the immune system, although it does not reflect the overall severity of the disease in PUUV infection.

Systemic suPAR levels are considered to reflect the degree of immunoactivation of the individual. This is corroborated by numerous studies showing suPAR levels to be increased as well in cancer as in various inflammatory and infectious diseases [22]. Furthermore, high suPAR concentrations are predictive of outcome and mortality in these conditions [22]. Previous studies on patients with infectious diseases have demonstrated that suPAR can predict disease severity and case fatality in malaria, tuberculosis, sepsis, bacterial meningitis, and some viral infections [24–27,29,30,40–44].

Previously, suPAR has been studied only in a few viral infections. However, in HIV infection, several studies show that suPAR is a strong predictor of immunologic failure and mortality [28,41]. In addition, the suPAR levels decrease with effective antiretroviral therapy [40]. In patients with Crimean-Congo hemorrhagic fever, suPAR is elevated and also predictive for mortality [42]. Systemic suPAR levels also predict the progression of liver fibrosis to cirrhosis in patients with chronic hepatitis C [45,46]. Furthermore, elevated suPAR levels have been found in the cerebrospinal fluid of patients with viral meningitis [44]. Here, we demonstrate that suPAR is elevated and associates with severity of the disease in PUUV-induced hantavirus infection.

The exact pathogenetic mechanisms in hantavirus infection are currently still unclear. Pathological changes are marked by increased capillary permeability in the affected organs, which also explains many signs and symptoms in these infections [11–13].

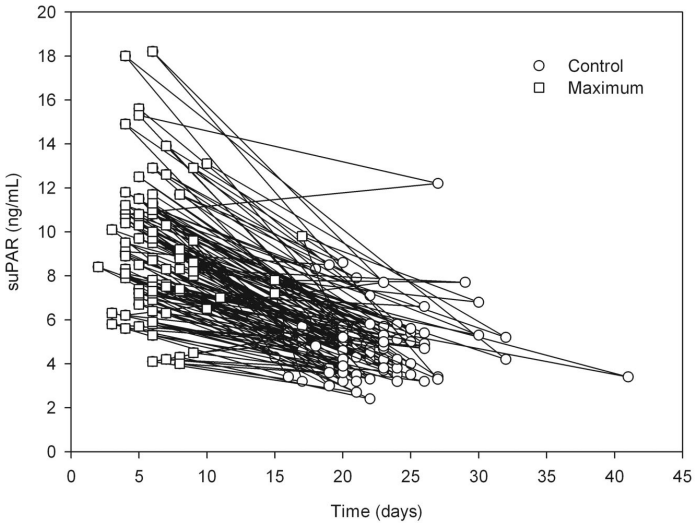

Figure 1. Line chart showing soluble urokinase-type plasminogen activator receptor (suPAR) maximum (median 8.7 ng/ml, range 4.0–18.2 ng/ml) and convalesce phase concentrations (median 4.7 ng/ml, range 2.4–12.2 ng/ml) in relation to the onset of fever (day 0) in 97 patients with Puumala hantavirus infection (P-value for the difference <0.001). Short title: Line chart showing suPAR maximum and convalescence concentrations in relation to the onset of fever.
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The endothelium of the small vessels in various organs is the primary target in hantavirus infection, and the cellular entry of pathogenic hantaviruses is mediated by β_3-integrins [11,13–15]. Integrins are heterodimeric surface receptors on endothelial cells and platelets, mediating cell-to-cell adhesion, cell migration, extracellular matrix protein recognition, and platelet aggregation. β_3-integrins have an important role in regulating vascular integrity, endothelial cell permeability, and platelet functions [47]. Pathogenic hantaviruses probably interfere with these

Table 3. The correlations of maximum plasma suPAR levels with clinical and laboratory variables reflecting the severity of the infection in 97 patients with acute Puumala hantavirus infection.

	R	P-value
Length of hospitalization	0.325	0.001
Change in weight during hospitalization	0.406	<0.001
Urinary output min	−0.332	0.002
Creatinine max	0.378	<0.001
Platelets min	−0.325	0.001
Hematocrit min	−0.369	<0.001
Leukocytes max	0.475	<0.001
CRP max	0.298	0.003
PTX3 max	0.425	0.005
IL-6 max	0.621	<0.001
IDO max	0.557	<0.001
cf-DNA max	0.363	0.018

Min = minimum, Max = maximum, CRP = C-reactive protein, PTX3 = pentraxin-3, IL-6 = interleukin-6, IDO = indoleamine 2,3-dioxygenase, cf-DNA = cell-free DNA.
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Table 4. Maximum plasma suPAR levels in different patient groups in 97 patients with Puumala virus infection.

	suPAR levels		
	Median	Range	P-value
Sex			0.841
Male	8.6	4.2–18.2	
Female	8.8	4.0–15.3	
Dialysis			0.038
Yes	10.8	8.6–18.0	
No	8.4	4.0–18.2	
Hospital stay			0.007
>6 days	9.2	6.5–18.2	
≤6 days	8.3	4.0–13.1	
Minimum platelet level			0.019
$<50\times10^9$/l	9.7	5.7–18.2	
$\geq50\times10^9$/l	8.2	4.0–15.6	
Maximum leukocyte count			<0.001
$>10\times^9$/l	9.2	5.7–18.2	
$\leq10\times^9$/l	7.5	4.0–13.1	
Maximum creatinine			0.001
>200 μmol/l	9.4	6.5–18.2	
≤200 μmol/l	8.0	4.0–15.6	

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functions, hence increasing endothelial permeability [47]. uPAR, in turn, interacts with integrins, including β_3–integrins, and is believed to regulate their activation degree by altering their adhesive properties and signaling capacity [21,22].

In this study, we found that plasma suPAR is markedly elevated in patients with PUUV-infection. Moreover, the higher the suPAR level is, the severer is also the disease. Taken into account the role of β_3-integrins in the course of hantavirus infection, and on the other hand, the interactions of (s)uPAR and integrins, our finding presents as interesting. It brings up the possibility that increased suPAR is involved in the pathogenesis of PUUV infection by activating β_3-integrins.

Conclusions

The plasma level of suPAR is elevated in acute PUUV infection and associates with the severity of the disease. Therefore, plasma suPAR determinations may offer a potential diagnostic tool for assessing the severity and outcome of the disease. In addition, it is possible that circulating suPAR is involved in the pathogenesis of PUUV-induced hantavirus infection by activating β_3-integrins. However, future studies are warranted to verify this assumption.

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Author Contributions

Conceived and designed the experiments: TKO LT SM RH NM HH AV JM JA. Performed the experiments: NM JA. Analyzed the data: TKO SM. Contributed reagents/materials/analysis tools: JA. Wrote the paper: TKO. Produced the figure: HH. Drafted the article or revised it critically: TKO LT SM RH NM HH AV JM JA. Approved the version to be published:TKO LT SM RH NM HH AV JM JA.

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