Cardiopulmonary involvement in Puumala hantavirus infection

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To my beloved wife

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Abstract

Puumala hantavirus (PUUV) causes hemorrhagic fever with renal syndrome in Europe. After inhalation of virus shed by bank voles, the virus systemically targets the vascular endothelium leading to vascular dysfunction and leakage. Many patients with PUUV infection experience cardiopulmonary manifestations but the underlying mechanisms have not been determined.

The aims of the studies presented were to describe cardiopulmonary manifestations, investigate pathogenetic mechanisms including presence of virus in the lungs and the local immune response in PUUV infection.

The results showed cardiopulmonary involvement of varying severity in almost all studied patients. High-resolution computed tomography frequently revealed vascular leakage into the lungs or pleural cavities. Pulmonary function tests generally showed reduced gas diffusing capacity, evidenced in patients as dyspnea, poor oxygenation and frequent need of oxygen treatment. Among patients who were not fully recovered at 3 months follow-up, remaining decreased gas diffusing capacity was highly common.

Echocardiography revealed mainly right heart dysfunction which was related to manifestations within the lungs, in terms of increased estimated pulmonary vascular resistance, mild to moderate pulmonary hypertension, and reduced right ventricular systolic function in patients with more pronounced lung involvement, as indicated by need of oxygen treatment.

Analyses on bronchoalveolar lavage (BAL) and bronchial biopsies revealed a highly activated cytotoxic T cell (CTL) response in the lungs. The CTL response was not balanced by the expansion of regulatory T cells and high numbers of CTLs were associated with more severe disease. PUUV RNA was detected in almost all patients' BAL samples and the viral load was inversely correlated to the number of CTLs.

Three patients presenting with severe and fatal cardiopulmonary distress were also described. Autopsies revealed PUUV protein in vascular endothelium in all investigated organs, including the heart and lungs, along with a massive CTL response mainly in the lungs.

In conclusion, cardiopulmonary involvement of varying severity was present in almost all patients with PUUV infection. Cytotoxic immune responses could contribute to disease development but also help in clearing the infection. Long lasting fatigue after hantavirus infection may be explained by remaining manifestations within the lungs.

Populärvetenskaplig sammanfattning

Sorkfeber tillhör sjukdomsgruppen blödarfebrar och orsakas av Puumalavirus, ett hantavirus. Runt om i världen finns ett flertal sjukdomsalstrande hantavirus, och alla dessa har sin egen gnagarvärd. Skogssorken utgör reservoaren för Puumalavirus. Värddjuret utvecklar inga tecken på sjukdom men bär på en kronisk infektion med periodvis utsöndring av virus i urin, avföring och saliv. Människan smittas oftast genom inandning av damm innehållande viruspartiklar. Viruset infekterar cellerna i de minsta blodkärlens väggar, och efter en inkubationstid på vanligen 2-3 veckor bryter sjukdomen ut. Hantavirusinfektion kan ge upphov till två allvarliga kliniska syndrom: blödarfeber med njurpåverkan (HFRS) orsakat av hantavirus i Europa och Asien, samt hantavirussjukdom med hjärt- och lungpåverkan (HCPS) orsakat av hantavirus i Nord och Sydamerika. HFRS är mest känt för övergående njursvikt och i viss mån blödningar, medan HCPS ofta leder till svår hjärt- och lungpåverkan med hög dödlighet. Kännetecknande för både HFRS och HCPS är kraftig inflammation och störd funktion i blodkärlsväggen, ledande till läckande kärl och ödem i vävnaderna.

Hantavirussjukdom orsakas i Sverige, så vitt man vet, bara av Puumalavirus och leder till en mildare form av HFRS även kallad för sorkfeber eller nephropathia epidemica. Dödligheten är låg (<0,5 %), men sjukdomen orsakar betydande lidande för patienten. Den akuta sjukdomsperioden omfattar cirka 1-2 veckor, och ungefär var tredje patient behöver vårdas inneliggande på sjukhus. Efter den akuta sjukdomsperioden besväras många patienter med sorkfeber av trötthet som långsamt avtar under de kommande månaderna. Förutom övergående njurskada får patienter med sorkfeber ofta symtom från lungorna i form av hosta och andfåddhet, samt från hjärta och kärl i form av avvikande hjärtrytm och blodtrycksfall. Hjärt- och lungengagemanget vid sorkfeber liksom de bakomliggande mekanismerna, är ofullständigt undersökta. Studier har visat att ett överdrivet starkt immunsvar, delvis bestående av så kallade cytotoxiska T-celler, kan vara sjukdomsalstrande vid hantavirussjukdom.

Målsättningen för studierna som avhandlingen baseras på var att karaktärisera hjärt- och lungengagemanget vid sorkfeber och beskriva möjliga bakomliggande mekanismer, inklusive förekomst av virus i lungorna och det lokala inflammatoriska svaret.

Resultat från studierna visade hjärt- och lungpåverkan av varierande svårighetsgrad hos nästan alla studerade patienter. Högupplöst skiktröntgen visade vätskeläckage i lungor och lungsäck hos 46 % av patienterna. Lungfunktionsundersökning visade att sorkfeber generellt ledde till nedsatt

gasutbyte i lungorna, vilket medförde dålig syresättning, andfåddhet och behov av tillfällig syrgasbehandling hos en tredjedel av de studerade patienterna.

Vid uppföljning efter tre månader uppgav hälften av patienterna att de inte var återställda, vilket yttrade sig som trötthet eller andfåddhet vid ansträngning. Lungfunktionen hade förbättrats hos samtliga men var fortfarande onormal i 38 % av fallen. De som inte upplevde sig återställda efter tre månader hade tydligt sämre lungfunktion jämfört med dem som kände sig återställda.

Ultraljudsundersökning av hjärtat visade vanlig förekomst av lindrigt till måttligt påverkad hjärtfunktion. Detta var mest tydligt på högersidan av hjärtat och syntes relaterat till förändringar inom lungorna med uppskattat ökat motstånd i lungkretsloppet. Det ledde till ett tyngre arbete för höger kammare och synbart sämre pumpförmåga hos de patienter som hade mer uttalat lungengagemang i form av behov av syrgasbehandling.

Bronkoskopier genomfördes under den akuta sjukdomstiden med provtagning i form av bronksköljningar och vävnadsprover från luftvägarna. Efterföljande analyser visade på ett höggradigt aktiverat immunsvar i nedre luftvägarna, huvudsakligen bestående av ett ökat antal cytotoxiska T-celler. Det starka immunsvaret i lungorna balanserades inte av ett ökat antal regulatoriska T-celler, vilket skulle kunna tyda på en okontrollerad inflammatorisk reaktion. Patienter med ett kraftigare cytotoxiskt immunsvar uppvisade flera tecken på svårare sjukdom. Puumalavirus hittades i bronksköljvätska hos nästan alla patienter, och virusbördan visade sig vara omvänt proportionell mot antalet cytotoxiska celler i sköljvätskan. Detta skulle kunna tala för att de cytotoxiska cellerna hjälper kroppen bekämpa infektionen. Som stöd för denna tanke hittades också höga nivåer av proteiner som cytotoxiska celler utsöndrar för att inducera programmerad celldöd i infekterade celler.

Tre patienter var kritiskt sjuka och uppvisade svår hjärtlungpåverkan, vilket ledde till dödlig utgång i två fall. Vid obduktion hittades Puumalavirusprotein i kärlväggarna i alla undersökta organ, inklusive hjärta och lungor, tillsammans med ett massivt cytotoxiskt immunsvar framför allt i lungorna.

Sammanfattningsvis hittades hjärtlungpåverkan med varierande svårighetsgrad hos nästan alla patienter med sorkfeber. Cytotoxiska T-celler kan bidra till sjukdom vid sorkfeber, men de kan också hjälpa kroppen besegra infektionen. Långvarig trötthet och andfåddhet efter sorkfeber kan förklaras av nedsatt lungfunktion även efter tre månaders konvalescens.

Abbreviations

ANDV	Andes virus
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
CD	Cluster of differentiation
CRP	C-reactive protein
CTL	Cytotoxic T cell
DLCO	Diffusing capacity of the lung for carbon monoxide
ECG	Electrocardiography
HCPS	Hantavirus cardiopulmonary syndrome
HLA	Human leukocyte antigen
HFRS	Hemorrhagic fever with renal syndrome
HRCT	High-resolution computed tomography
HTNV	Hantaan virus
IL	Interleukin
IVRT	Isovolumic relaxation time
LDH	Lactate dehydrogenase
NK cell	Natural killer cell
NT-proBNP	N-terminal pro-B-type natriuretic peptide
PUUV	Puumala virus
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SNV	Sin Nombre virus
TNF-α	Tumor necrosis factor-α

Original Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Rasmuson J, Andersson C, Norrman E, Haney M, Evander M, Ahlm C
Time to revise the paradigm of hantavirus syndromes? Hantavirus pulmonary syndrome caused by European hantavirus.
Eur J Clin Microbiol Infect Dis. 2011 May; 30(5): 685-690.

II Rasmuson J, Pourazar J, Linderholm M, Sandström T, Blomberg A, Ahlm C
Presence of activated airway T lymphocytes in human Puumala hantavirus disease.
Chest 2011 Sep; 140(3):715-722

III Rasmuson J, Lindqvist P, Sörensen K, Hedström M, Blomberg A, Ahlm C
Cardiopulmonary involvement in Puumala hantavirus infection
BMC Infect Dis. 2013 Oct; 13:501

IV Rasmuson J, Pourazar J, Mohamed N, Lejon K, Evander M, Blomberg A, Ahlm C
Cytotoxic immune responses in the lungs correlate to disease severity in patients with hantavirus infection.
Manuscript.

“I *knew* it. Those little bastards!”

Anonymous patient, 2003

Introduction

History of hantaviruses

One hundred years ago this year, medical officers reported home from the fronts in France of an enigmatic febrile disease defined by renal impairment, abdominal pain, dyspnea, and an irritating cough, that was hypothesized to be caused by an as of yet undetectable microorganism [1]. Hantaviruses, the topic of this thesis, were later identified and still cause disease in Europe and elsewhere.

Hantavirus forms a separate genus in *Bunyaviridae*, a family of viruses causing potentially severe infections in humans, animals and even plants worldwide. Human disease associated with important viruses in the *Bunyaviridae* family includes zoonotic hemorrhagic fevers and meningoenchephalitis [2]. Hantaviruses have a negative-sense single-stranded RNA genome, consisting of three segments (named small, medium, and large), encoding a nucleocapsid protein, two envelope glycoproteins, and a RNA-dependent RNA polymerase, respectively [2, 3]. Various rodents, shrews, moles and bats act as reservoirs, with each animal species carrying its own specific hantavirus. Although, hantaviruses pathogenic to humans have so far only been identified in rodents [4-6]. The virus shows high resistance to environmental conditions, as it can be infectious for two weeks at room temperature and even longer in cooler conditions [7].

Infections caused by hantavirus may have been described in Chinese medical literature as far back as 960 AD [8]. Full awareness of hantavirus disease reached Western medical science community when reports were published concerning hemorrhagic fever among United Nations troops in Korea during the war in the early 1950s (named epidemic hemorrhagic fever) [9]. Previously, milder but similar disease was reported in Europe during the First World War (termed trench nephritis) [1, 10] and in Sweden in the 1930s (called nephropathia epidemica) [11, 12]. Preceding the outbreaks of hemorrhagic fever in Korea in the 1950s, the disease was encountered and described in East Siberia in the 1930s and 1940s and by the Japanese army in Manchuria during the same time period. Russian and Japanese scientists independently performed extensive studies of hemorrhagic fever involving interhuman inoculation experiments in “volunteers”, best described as unrecognized crimes against humanity (referred by [13-20] and [21, 22]).

In the late 1970s, with the discoveries of Hantaan virus (HNTV, hantavirus genus prototype species) in Korean striped field mice (*Apodemus agrarius*) and Puumala virus (PUUV) in Finnish bank voles (*Myodes glareolus*), the causative agents of Asian and European hemorrhagic fever could be

determined [23, 24]. The first (and later assigned) hantavirus, Thottapalayam virus, was discovered already in India in 1964 in an Asian house shrew (*Suncus murinus*) [25] but is not considered to cause disease in humans. An outbreak in southwestern United States in 1993 of a mysterious febrile illness leading to acute respiratory failure led to the discovery of Sin Nombre virus (SNV) [26, 27], and subsequent detection of multitudes of other hantaviruses throughout the Americas [4, 28].

To date, according to the International Committee on Taxonomy of Viruses 2013 release available on their website (www.ictvonline.org), 24 hantavirus species have been officially recognized, out of which 11 have been convincingly shown to cause disease in humans (Table 1). Hantavirus phylogeny is growing ever more complex with new viruses discovered as potential carrier animals are investigated. Numerous newly described viruses, many with uncertain status as human pathogens, have yet to receive official recognition [6, 29-31]. The most important hantaviruses known to cause disease in humans are HNTV in Eastern Asia, PUUV and Dobrava-Belgrade virus in Europe including Western Russia, Sin Nombre virus (SNV) in North America, Andes virus (ANDV) and related viruses in South America, and Seoul virus worldwide [4, 5, 28]. In contrast to all other members of the Bunyavirus family, hantaviruses have not been shown to be transmitted to humans by an arthropod vector [2]. Instead, transmission of hantaviruses to humans is believed to mostly occur by inhalation of dust containing virus shed in urine, feces and saliva from persistently infected rodents [4, 32, 33]. Common activities associated with increased risk of human hantavirus infection include handling firewood, cleaning (often inside buildings unused for a period of time, e.g. summer cottage), forestry work, farming, and camping [34-38]. Smoking appears to increase the risk of hantavirus infection and thereby further suggest importance of respiratory transmission and the condition of the airway [38]. Other less frequent routes of transmission have also been reported, including person-to-person infections among household and health care contacts in ANDV infections in Argentina and Chile [39-44], bites by rodents [45], and blood product transfusions [46].

	Virus species	Reservoir	Geographic distribution	Disease
Old World				
	Dobrava-Belgrade virus	*Apodemus flavicollis*	Europe	HFRS
	Hantaan virus	*Apodemus agrarius*	Asia	HFRS
	Khabarovsk virus	*Microtus fortis*	Asia	Unknown
	Puumala virus	*Myodes glareolus*	Europe	HFRS
	Saaremaa virus	*Apodemus agrarius*	Europe	HFRS
	Sangassou virus	*Hylomyscus simus*	Africa	Unknown
	Seoul virus	*Rattus rattus and R. norvegicus*	Worldwide	HFRS
	Thailand virus	*Bandicota indica*	Asia	Unknown
	Thottapalayam virus	*Suncus murinus*	Asia	Unknown
	Topografov virus	*Lemmus sibericus*	Asia	Unknown
	Tula virus	*Microtus arvalis*	Europe	Unknown
New World				
	Andes virus	*Oligoryzomys longicaudatus*	South America	HCPS
	Bayou virus	*Oryzomys palustris*	North America	HCPS
	Black Creek Canal virus	*Sigmodon hispidus*	North America	HCPS
	El Moro Canyon virus	*Reithrodontomys megalotis*	North America	Unknown
	Isla Vista virus	*Microtus californicus*	North America	Unknown
	Laguna Negra virus	*Calomys laucha*	South America	HCPS
	Muleshoe virus	*Sigmodon hispidus*	North America	Unknown
	New York virus	*Peromyscus leucopus*	North America	HCPS
	Prospect hill virus	*Microtus pennsylvanicus*	North America	Unknown
	Rio Mamore virus	*Oligoryzomys microtis*	South America	Unknown
	Rio Segundo virus	*Reithrodontomys mexicanus*	South America	Unknown
	Sin Nombre virus	*Peromyscus maniculatus*	North America	HCPS

Table 1. Recognized hantavirus species according to the International Committee on Taxonomy of Viruses 2013 release combined with data from references [4, 47-49].

Puumala virus is the only detected hantavirus in Sweden, causing up to 2200 notifiable cases annually (median around 300), according to statistics from the Public Health Agency of Sweden (www.folkhalsomyndigheten.se). Infection with PUUV is principally restricted to central and northern Sweden [50, 51]. A vast majority of diagnosed patients are between 35-74 years old (people in all age groups are susceptible). There is a slight preponderance of males among diagnosed cases (1.5:1 male:female ratio) [52, 53], but epidemiological studies have shown similar seroprevalence in both sexes [54]. Recognized cases represent the tip of the iceberg, as studies have indicated that approximately seven out of eight Swedish PUUV infections go undiagnosed [54], suggesting the likelihood of frequent mild or even asymptomatic infections.

Hantavirus infections in the animal host

It is generally believed that hantavirus infection in the animal hosts is chronic and without visible signs of disease [4, 5, 55]. It could be questioned whether the infection is truly asymptomatic and persists without inducing pathology in the host. Infections with wild-type hantaviruses in their respective rodent host (both in captivity and in the wild) has been shown to induce low grade pathological changes, most often in the lungs (e.g. edema or small hemorrhagic inflammatory pulmonary infiltrates) [56-58], and may lead to long term negative health consequences for infected animals (e.g. reduced winter survival) [59, 60]. Nevertheless, hantavirus infections in carrier animals persists, contrary to humans, seemingly for the rodents' lifespan and causes infections that perhaps at the most could be labeled paucisymptomatic. How this mode of persistence can be achieved has drawn considerable attention, since understanding the mechanisms behind it could help us understand what makes the human body clear the infection, although at the cost of a potentially lethal illness. It has been suggested that hantaviruses may achieve persistence by evasion from, or suppression of, host immune responses [55, 58, 61]. Indeed, there is growing evidence that hantaviruses possesses such abilities [58, 62-65].

Hantavirus infections in humans

Initial symptoms and laboratory findings

The incubation period for hantavirus infection is most often 2 to 3 weeks but may range from 1 to 6 weeks in occasional patients [43, 66-69]. Onset of hantavirus disease is usually abrupt with an influenza-like febrile prodrome, including a variation of symptoms such as fever, malaise, weakness, myalgia, headache, backache, nausea, vomiting, abdominal pain, and diarrhea [16, 27, 70-76]. A principal characteristic of hantavirus disease is vascular dysfunction, leading to capillary leakage syndrome and development of hypotension and edema [5, 27, 77-79]. Routine laboratory investigations in blood may detect multitudes of abnormalities indicating a generalized infection with systemic inflammatory response. Typical findings include thrombocytopenia and elevated D-dimer as signs of coagulopathy, inflammation in terms of leukocytosis and high C-reactive protein (CRP), acute kidney injury evidenced by increased creatinine and hematoproteinuria, elevated lactate dehydrogenase (LDH) suggesting cell damage, and elevated liver transaminases as signs of hepatitis. Hemoconcentration (e.g. increased hematocrit or hemoglobin concentration) and hypoalbuminemia are seen as signs of vascular leakage, also suggested by detection of low circulating plasma volume and patients

complaining of thirst. Later during the disease, reduced hematocrit is considered to reflect fluid overload. Electrolyte disturbances are common, with hyponatremia being most frequently found [16, 27, 70, 71, 74, 78, 80]. Over the following days from debut of the febrile prodromal phase, disease progression varies mainly depending on the infecting virus genotype [4]. There is widespread consensus that surviving hantavirus infection provides life-long immunity to the particular virus genotype, or possibly even against heterotypic hantaviruses [5, 81, 82]

Hantavirus syndromes

After the initial febrile prodromal phase, infections with hantaviruses found in the Old World (Europe and Asia) and New World (the Americas) show major differences in dominant organ manifestations and severity. The disease caused by hantaviruses in Europe and Asia is prominently characterized by coagulopathy and acute renal impairment and thereby named hemorrhagic fever with renal syndrome (HFRS) [1, 11, 12, 16, 70, 80, 83]. However, American hantaviruses instead often cause infections that lead to severe cardiopulmonary failure, hence titled hantavirus cardiopulmonary syndrome (HCPS, also known as hantavirus pulmonary syndrome) [27, 74, 75]. Although these clinical syndromes may appear as two separate entities, the disease caused by Eurasian or American hantaviruses also show many similarities, making some authors suggest that hantavirus syndromes could in large part be seen as varying clinical presentations of a similar underlying pathogenesis [5, 84-86].

Hemorrhagic fever with renal syndrome

HFRS has been described to progress through five distinct phases, named febrile, hypotensive, oliguric, polyuric, and convalescent, with each phase lasting hours to days (Figure 1) [83, 87]. The phases are more easily recognized in severe cases whereas the transitions may be difficult to identify in milder cases. Disease debut typically occurs with the mentioned febrile prodrome that may last up to 7 days. At the end of the febrile phase, a mild to severe capillary leakage syndrome develops leading to varying degree of edema in central organs and periphery, preferentially in the retroperitoneal space [77, 83, 87-89]. Patients may suffer from lightheadedness and hypotension, with occasional fainting and risk of hypotensive shock in severe cases. Most fatalities occur in this stage of disease [90, 91]. During the following days patients develop progressive indications of renal involvement, as indicated by excessive protein and blood in urine, decreased urine production (oliguria <400 mL urine/24 h; anuria <100 mL urine/24 h), elevation of blood markers indicative of acute kidney injury (e.g. urea and creatinine), and tenderness over kidneys. Around 8-10 days post onset of

fever, renal failure peaks and the patient enters a polyuric phase with large volumes of urine production before normalization and the patient enters convalescence with gradual improvements until full recovery during the following weeks to months. Aside from this classic and brief syndrome description, patients may encounter many other more or less frequent complications to the clinical course, including blurred vision in approximately one-third of patients [71, 73, 87, 92-94], respiratory manifestations [71, 73, 90, 91, 95-98], appendicitis [99], myocarditis [73, 77, 100-102], pancreatitis or acalculous cholecytisis [91, 102, 103], hypophyseal injury leading to hormonal insufficiency [77, 104-106], encephalitis [90, 92, 107-110], epileptic convulsions [87, 90, 92, 93, 110], and Guillain-Barré syndrome [111-114]. Contrary to the somewhat misleading syndrome name, clinically apparent bleedings range from rare in PUUV infection [115] to infrequent in HTNV infection [90] and are, when present, most often harmless (e.g. epistaxis). Instead, thrombotic disease may pose a larger threat to health when considering the coagulopathic complications during and after hantavirus infection [116, 117].

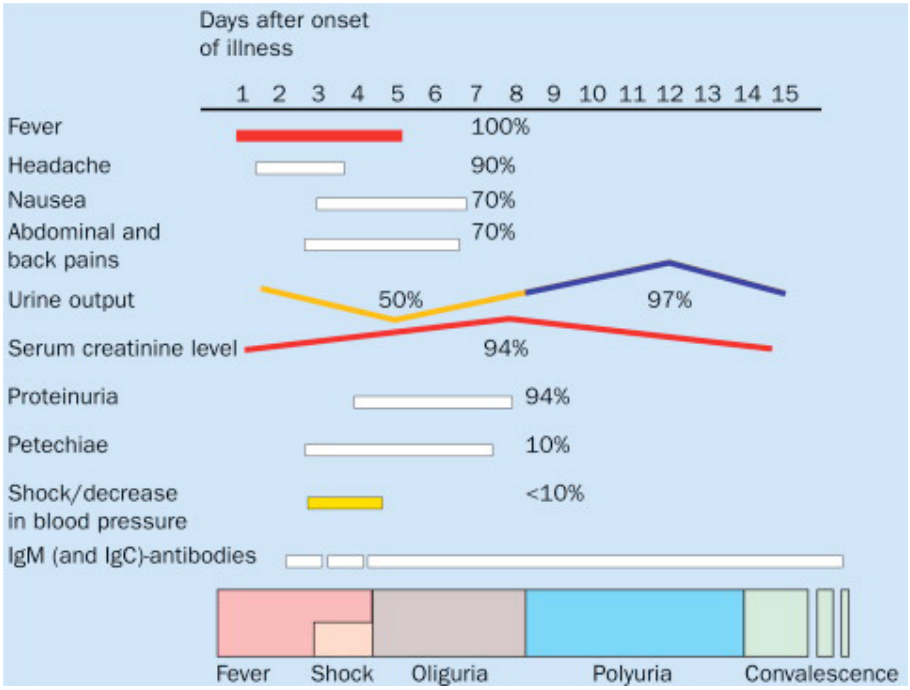

Figure 1. Schematic representation of typical clinical course of Puumala virus infection (Vapalahti et al. 2003 [83]). Figure reprinted with kind permission from the publisher.

Worldwide, most cases of hantavirus disease are diagnosed in China (HTNV and Seoul virus), where up to 115 000 HFRS cases were reported in peak years in the 1980s, dropping to 10-50 000 annual cases in the last decade, perhaps the result of a successful vaccination campaign [118]. In Europe, western Russian provinces report most cases (peak years >10 000, median 3000 cases). Other countries reporting significant numbers of (median annual) HFRS cases are Finland (1000), Sweden (300), Germany (100), France (100), Belgium (100), Norway (50), and Slovenia (15) [83, 86]. Infections occur all year round but most infections occur during autumn and early winter showing a 3-4 year peak-cycle coinciding with maximum rodent population [37, 119]. In contrast to the often high morbidity in HFRS and the lack of specific treatment, modern overall case fatality rates are very low for PUUV and Seoul virus infections (<0.5-1%), but increase to 1-5% in HTNV infections and even up to 10% in disease caused by Dobrava-Belgrade virus [52, 53, 119, 120]. This describes a "virus-gradient" determining disease severity, but when viewing individual cases caused by PUUV, Seoul virus, HTNV or Dobrava-Belgrade virus, these may vary from being paucisymptomatic febrile to fulminant and lethal [4, 72, 87]. The cause of death in acute HFRS has (in falling order) been attributed to irreversible cardiogenic shock, pulmonary edema, complications of renal failure, and ill-sited bleedings [72, 90, 91, 100, 111, 121].

Most of the information concerning autopsy findings in fatal HFRS comes from the extensive studies performed in Korea during the early 1950s [77, 88, 90, 122]. In addition, concurring results can be gathered from the rare case reports concerning mortal PUUV infection [100, 104, 111, 121]. Necropsy results were in Korean HFRS described to vary depending of time of death in respect to disease progression. Most pathological findings could be related to vascular dysfunction that was described as severe to extreme in patients dying in hypotensive shock. In these patients, vascular congestion with extravasation of erythrocytes, interstitial edema and hemorrhages were found mainly in the kidneys, in the heart, and in the hypophysis. The hearts have occasionally been dilated and flabby, but were in almost all cases of normal size and weight. Necrosis has commonly been observed in hypophysis and kidneys. Vascular effusions have been described as most extreme in kidneys and the bulging retroperitoneal space, and less so to the lung parenchyma, abdomen, and pleural and pericardial cavities. Similar but less severe findings were reported in patients dying during oliguric or polyuric phases. Here, signs of vascular leakage were instead found in heavy edematous lungs occasionally exhibiting initial bacterial superinfection, while retroperitoneal edema was virtually absent [77, 88, 90, 122].

Hantavirus cardiopulmonary syndrome

Hantavirus cardiopulmonary syndrome was first recognized in 1993 in the United States and caused by SNV. Patients presented with the mentioned febrile prodrome that was followed by a rapidly progressing pulmonary edema leading to respiratory failure, along with often-fatal cardiogenic shock [27, 74, 123-126]. The pulmonary edema has been characterized as noncardiogenic, meaning related to increased capillary permeability and not caused by left ventricular heart failure, indicated by normal pulmonary capillary wedge pressures and edema protein content similar to plasma [27, 123, 124]. Indications of renal involvement (elevated creatinine levels, hematuria, proteinuria, or oliguria) and coagulopathy were generally present but reported to rarely be of clinical significance [27, 34]. Later, as more New World hantaviruses were described, the difference between HCPS and HFRS became more diffuse when prominent renal impairment and bleeding manifestations were found to be common in HCPS caused by other North and especially South American hantaviruses [28, 75, 127-129]. Similar to HFRS, HCPS has been described to occur in several distinct phases [4, 130]. Patients with HCPS experience respiratory manifestations such as cough (40-100% of patients), shortness of breath (90-100%), desaturation and need of supplemental oxygen treatment (50-100%) [27, 34, 74, 75, 128]). Intubation and mechanical ventilation is required in up to 60% of cases [125]. Similar to patients with acute respiratory distress syndrome (ARDS) [131-133], patients with HCPS displayed increased pulmonary vascular resistance and pulmonary artery hypertension [124]. However, contrary to ARDS, histopathology revealed no alveolar epithelial destruction, hyperplasia of type II pneumocytes, neutrophil infiltration, or widespread hyaline membranes, suggesting differences in pathogenesis [34].

There is evidence cardiac involvement in HCPS is functional rather than structural, although there are few comprehensive reports of heart physiology, including studies of hemodynamics and electrocardiograms (ECG). Myocardial depression has been reported to be central in HCPS and is by cardiogenic shock along with severe arrhythmias responsible for most deaths [27, 28, 34, 75, 123, 124, 134]. Markers for myocardial cell death (troponins or creatine kinase MB fraction) were normal or only slightly elevated [27, 74]. Furthermore, patients with HCPS that survive the first days of cardiopulmonary phase, despite life-threatening hypotensive shock, may recover and normalize cardiac function as quickly as they deteriorated [123]. Echocardiographic data from patients with HCPS is scarcely described in papers also containing more informative pulmonary artery catheter data. These reports have shown that HCPS patients display low stroke volume and cardiac indices that only modestly improve by intravenous fluid therapy or inotropic drugs, along with normal or increased systemic vascular resistance

[27, 75, 123, 124]. The findings were noted being in contrast to the hemodynamic state in most patients with septic shock, where an elevated cardiac index is seen along with reduced systemic vascular resistance [135-137]. This made authors suggest that HCPS patients suffer from more pronounced myocardial depression than patients with septic shock of other cause [123, 124, 138]. Similar results were shown in the lethal hamster model for HCPS [139]. The mechanisms of myocardial depression may be attributed to high expression of proinflammatory cytokines in the myocardium, including tumor necrosis factor (TNF)-α and interleukin (IL)-6, described both in human and hamster HCPS [138, 140]. Similarly, TNF-α and IL-6 contribute to myocardial depression in sepsis [141-144] and TNF-α was suggested to induce hypotension in HFRS [145, 146].

Autopsies in patients dying in HCPS have, as in fatal HFRS, revealed evidence of profound vascular leakage. However, contrary to HFRS, the leakage was almost entirely localized to the lungs (described as rubbery, heavy, and airless) and pleural cavities (effusions up to 8 L) with grossly normal findings in other organs [34, 134, 138]. Similar to some fatal HFRS cases, biventricular dilatation with overstretching of myocardium and interstitial edema has been reported in Brazilian HCPS, however, without the intracardial hemorrhages seen in Korean HFRS [77, 138]. In contrast, hearts from SNV infected patients have been described as grossly and histologically normal [134], further reflecting clinical variations in disease caused by different hantaviruses.

As in HFRS, there is a large variation in disease severity comparing infections with different HCPS-related hantaviruses, as well as between individual cases that may range from mild, or asymptomatic [147-150], to critical [27, 75]. Altogether, close to 5000 cases of HCPS have been reported in the Americas since 1993 with a case fatality rate around 40% [28, 125].

Cardiopulmonary involvement in HFRS

Mild to moderately severe pulmonary manifestations in patients with HFRS were frequently reported already in the descriptions of hemorrhagic nephritis in First World War and Korean War troops, but have often been overshadowed by the dominant and almost constantly present renal involvement. Symptoms from the lungs, in terms of cough and dyspnea, have been reported in up to 40% of patients with HFRS [1, 70-73, 76, 80, 151]. Severe respiratory manifestations, presenting as acute respiratory distress syndrome (ARDS) also occur and have been reported in 5-10% of HFRS patients in Korea and China and with case fatality rates up to 50% in those individuals [77, 87, 90, 91, 152]. Most of our understanding of respiratory involvement in HFRS, and what possibly causes it, comes from clinical

studies during Korean War in the 1950s and later in European PUUV infected patients mainly from the early 1990s and forward.

Studies to characterize pulmonary involvement in PUUV infection revealed chest abnormalities by standard X-ray or computed tomography in 16-28% and 53-92% of patients, respectively [16, 73, 95, 96, 153]. Pathological changes, similarly seen in Korean HFRS [154], were mainly thoracic effusions, interstitial infiltrates or pulmonary edema that were mostly related to hypoproteinemia, weight gain, and leukocytosis [95, 96]. Additionally, reduced lung gas exchange by lowered diffusing capacity was reported in almost all investigated PUUV infected patients [98].

Several mechanisms have been considered responsible for pulmonary edema, pleural effusions, and impaired gas exchange, all signs of extravasation of fluid into the thoracic compartment. The main controversy has been whether leakage into lungs and pleural cavities in HFRS is due to capillary hyperpermeability, as present elsewhere and most prominently in the retroperitoneum, or if it caused by increased intravascular hydrostatic pressure due to fluid overload as a consequence of renal failure [77, 87, 89, 90, 95, 96, 152, 155]. The postulate of uremic injury to endothelium provides a sort of middle ground but with uncertain significance [16, 89, 96]. Intravascular fluid overload as a mechanism for vascular leakage into the lungs was in the early 1950s suggested as less likely, since patients developed pulmonary edema prior to onset of oliguria, or in continuous neutral or negative fluid balance during the oliguric phase. Furthermore, almost all patients with pulmonary edema exhibited normal cardiac size and venous pressure, and lacked edema elsewhere [77, 87, 90, 152]. However, vigorous fluid resuscitation may worsen the situation as infused fluid quickly leaves the circulation and maintaining fluid balance has been considered important to improve the prognosis [130, 156, 157], suggesting adverse contributory effects of fluid overload. Transient volume overload, as a result of edema redistribution, impacting a previously injured vascular bed has been suggested as a potential mechanism of late oliguric pulmonary edema in HFRS [72, 77, 90, 152, 155], similarly described also in idiopathic capillary leakage syndrome (Clarkson's disease) [158].

Papers describing single cases or case series concerning patients infected with HFRS-related viruses (mostly PUUV) displaying HCPS-like illness are increasing in numbers [96, 97, 100, 104, 108, 121, 159-177]. Many of the reported patients displayed only mild, or nonexisting, renal impairment, in contrast to the severe, early, and sometimes lethal cardiopulmonary involvement. The basis of this variation in clinical presentation is still unknown. Nevertheless, the reports reflect the wide spectrum of hantavirus disease caused by HFRS-related viruses. The observation of a connection

between leukocytosis and changes in the thoracic compartment [95, 96] could be seen as indications of immunopathology contributing to pulmonary manifestations in HFRS, as has been suggested in HCPS [34, 84, 178, 179].

Similar to respiratory involvement, cardiac manifestations have been noted in clinical studies of HFRS patients. Among these, the majority of investigations were performed in Korea in the 1950s, while studies involving PUUV infected patients are rare. The most common findings have been changes in the ECG, impaired cardiac function, relative or absolute bradycardia, and occasional reports of clinical myocarditis that in lethal cases were described as focal mononuclear cells infiltrates in the myocardium and endocardium [16, 73, 77, 100, 101, 122, 180]. Studies in patients with Korean HFRS have shown large variations in patients' hemodynamic state depending on disease progression [152, 181]. In the early febrile phase, patients were (in spite of high fever) exhibiting normal or only slightly elevated cardiac output that gradually decreased into the hypotensive phase. In parallel to findings in hypotensive HCPS patients, low cardiac output along with normal to high vascular resistance was seen in hypotensive HFRS patients. In the oliguric phase patients instead exhibited significantly increased cardiac output and occasionally also hypertension (described as a hyperdynamic state) before returning to normal circulation when polyuria started. Pathological ECG, including unspecific inverted T-waves, hyperkalemic amplified T-waves, sinus bradycardia or tachycardia, and prolongation of Q-T interval, were described to occur in half of the HFRS cases investigated during the Korean War [182].

There are almost no studies of heart physiology in PUUV infection. Two available echocardiographic studies reported only scarce information of left heart morphology and visually judged left ventricle performance, but without details on hemodynamics or right heart function. Most patients (84%) were reported to display normal heart function, while the rest showed signs of ventricle contraction abnormalities or slight pericardial effusion [98, 180]. Changes in the ECG have been described in 38-57% of patients with PUUV infection [16, 101, 180], displaying many similarities with the HFRS patients in Korea [182]. The most commonly reported abnormal findings were inverted T-waves and sinus bradycardia or tachycardia. Other less frequent findings included reversible S-T elevations, first-degree atrioventricular block, supraventricular and ventricular extrasystoles, nodal rhythm, and atrial fibrillation [16, 101, 180]. Troponins were not elevated when evaluated by low sensitivity assay [180].

Diagnosing hantavirus infection

When diagnosing hantavirus infections, the most difficult part is to raise the suspicion and include it in the differential diagnosis. Hantavirus disease caused by PUUV may be suspected by a combination of typical symptom presentation, positive epidemiology including potential exposure to rodent excreta, and characteristic laboratory results (e.g. thrombocytopenia and urine dipstick revealing proteinuria and/or hematuria, later elevated serum creatinine). Dipsticks are especially useful, as protein and blood are almost always detected in the urine [83], and they are cheap and readily available. Securing etiological diagnosis is normally done by serology (immunoflourecence or ELISA assays) determining virus-specific immunoglobulin M (IgM) and IgG that are present by disease onset or during the early febrile prodrome in almost all patients [24, 83]. Rapid serological tests [183] with > 95% sensitivity and specificity compared to standard serology are often used and may help avoid unnecessary antibiotics [184], as bacterial sepsis is a common differential diagnosis. Additional means to secure the diagnosis include detection of viral RNA in blood by reverse transcriptase (RT)-PCR that may be especially useful in severe hantavirus disease where humoral immune responses may be delayed [76, 185, 186].

Person-to-person transmission

Various body fluids, including blood, urine, respiratory secretion, and saliva, from humans with hantavirus infection have been shown to contain viral RNA and/or viral protein [43, 151, 176, 185, 187-192]. Aside from an occasional infection caused by blood transfusion [46], hantavirus transmission between humans has so far only been recorded in ANDV infections in Chile and Argentina [39-44]. A prospective evaluation of ANDV infected patients' household contacts has shown that almost one-fifth of contacts that had sex with an index case developed HCPS. However, infection was transmitted also to household contacts having a nonsexual relation to the index case [43]. Combined with reports of nosocomial infections and infections in other close social settings, such as car rides and offices, this suggests the possibility of limited airborne transmission of ANDV [40, 44]. Still, nosocomial infections appear rare, as ANDV seroprevalence among health care personnel working at hospital wards where HCPS patients were treated was not different from the seroprevalence of the general population [193, 194]. Contact with ANDV infected saliva has been suggested to be of special importance in transmission between humans [43, 151, 195, 196]. Interestingly, it has been shown that two hantaviruses without documented person-to-person transmission, PUUV and HTNV,

were inhibited by the antiviral proteins present in human saliva, while Andes virus remained infectious, possibly explaining its potential to transmit between humans [195].

Hantavirus pathogenesis

Several observations have lead to the proposition that hantavirus disease is mainly immune-mediated [4, 5, 85, 197]. Firstly, hantaviruses are noncytopathic both in cell cultures [198-202] and in human tissue [34, 77]. Secondly, hantavirus infection alone does not lead to symptoms, illustrated by viral replication in the long incubation period and temporal association of strong immune responses and onset of disease [43], in similarity with other noncytopathic viruses as hepatis B and C [203]. Thirdly, intense immune responses have been linked to more severe disease [73, 76, 87, 95, 204, 205]. The immunopathogenesis hypothesis has gained further support by findings of relations between disease outcome and host genetics linked to differences in intensity of immune responses (e.g. human leukocyte antigen [HLA] haplotype) [204, 206-210], but results are not conclusive. Hantavirus pathogenesis is likely a complex multifactorial process involving vigorous immune responses [85, 197], direct viral effects [211, 212], platelet dysfunction [211, 213], and viral immune evasion strategies [63-65]. Lack of relevant and practical animal models, at least for HFRS-related viruses, and reverse genetics have hampered experimental research in hantavirus pathogenesis.

Animal models

One major difficulty in research in hantavirus pathogenesis and treatment has been the absence of appropriate animal models that mimic human hantavirus disease. A few valuable models have been developed, including macaque models for PUUV and SNV infection [214-217] and Syrian hamster model for ANDV infection [82, 140]. Additionally, suckling mice and adult immunodeficient mice have been used to study HTNV infection, although these do not provide a model that resembles the human disease [218, 219].

Hantavirus distribution during infection

In blood

Viremia, defined by detection in blood of either infectious virus or viral genomes or proteins, is almost universally present in human hantavirus infections. The highest viral load is typically detected in the first sample early after disease onset, thereafter rapidly declining in following samples. Hantavirus genotypes related to more severe disease (e.g. SNV, HNTV or Dobrava-Belgrade virus) show 100-1000 times higher viral load compared to

species related to mild disease (PUUV). Duration of viremia, investigated in serum or plasma, lasts around 1-2 weeks regardless of hantavirus species [76, 185, 189, 192, 220, 221]. Evaluating whole blood samples, a much more protracted viremia could be detected, stretching up to 30 days post onset of fever for PUUV, 40 days for Dobrava-Belgrade virus, and >60 days for ANDV [43, 120], likely reflecting remaining infections in mononuclear cells [188, 221]. The magnitude of viremia was inversely correlated to the specific IgG response [76, 120, 220]. These observations indicate that the humoral immune response may effectively neutralize free virus in plasma, while cytolytic clearance of infected cells might be delayed, perhaps being a result of virus-conferred resistance against apoptosis to infected cells [63, 64]. A similar disease profile was also shown in the ANDV infection hamster model [82]. As the peak in viral load in blood seems to coincide with onset of disease or the first few days after fever debut, one would expect debut of viremia during the asymptomatic incubation period, especially considering its long duration. Accordingly, ANDV viremia was detected to precede onset of fever with 5-15 days, in a prospective study following exposed individuals [43].

In tissues and organs

Hantaviruses are believed to mainly use integrins, at least *in vitro*, to bind to and infect human cells [211]. It has been shown that pathogenic and apathogenic hantaviruses use different integrins (β3 and β1, respectively) [222, 223], perhaps reflecting differences in virulence. A wide range of immortalized or primary human cell lines (including endothelial cells, epithelial cells, and immune cells) representing targets believed to be infected by hantavirus *in vivo* have been shown to be susceptible to infection *in vitro*. In these cells, hantavirus infection causes no visible cytopathic effects [198-202]. *In vivo*, the primary target is believed to be the vascular endothelium in multiple organs, considering detected viral distribution and disease pathophysiology [34, 211, 219, 224]. The sequence of events and route by which hantaviruses may reach the endothelium after being inhaled remains unsolved. It has been suggested that bronchial and alveolar dendritic cells becomes infected with the inhaled virus, migrate from the lung to lymph nodes and thereafter spread the virus to endothelial cells and initiate immune activation [85, 225]. There are very few comprehensive studies investigating the simultaneous distribution of hantavirus in the human body and the majority of study subjects have been patients with HCPS caused by SNV or, to less extent, HFRS caused by HTNV. The vast majority of the viral antigen has been localized to the cytoplasm of vascular endothelial cells in capillaries and small blood vessels of investigated organs and tissues and, in the case of HTNV infection, also abundantly in the renal

epithelium [34, 134, 224]. The endothelium has been described as swollen, but without any other evidence of cytopathic viral effects [34, 77, 134, 226]. Additionally, various antigen presenting immune cells, in terms of spleen and lymph node follicular dendritic cells, lymphocytes, and macrophages (including liver Kupfer cells), have been shown to contain virus in patients with HCPS or HFRS [34, 134, 138, 176, 224]. It has been reported that SNV infections cause an exceptionally high viral load in the lungs (virtually every endothelial cell infected), relative to other infected organs [34, 134], similarly observed in a small case study of New York virus infection (HCPS agent) [227]. Myocardial and renal vasculatures were also reported to show prominent viral antigen staining, whereas the endothelium of liver sinusoidal vessels was only occasionally infected [34]. Given the respiratory route for transmission of hantaviruses, one would expect airway epithelium (bronchial epithelial cells or alveolar lining cells, pneumocytes) to be infected. However, studies evaluating this are exceedingly rare. In one study, New York virus was frequently found in alveolar lining pneumocytes and alveolar capillary endothelial cells, whereas no virus was detected in the bronchial epithelium [227]. In another study of fatal HCPS, alveolar epithelial cells were reported to contain most of the detected ANDV antigen, relative to the also infected alveolar endothelium. Interestingly, the authors also reported detection of viral antigen in secretory cells of patients' salivary glands [196]. In heart samples from Brazilian HCPS patients, viral antigen was detected in myocardial endotheliun and interstitial macrophages, along with an intense TNF-α staining in myocytes and macrophages [138]. Renal glomerular and tubular epithelial cells have shown abundant viral staining in biopsy or autopsy samples from HFRS patients (HTNV and PUUV), whereas this was only rarely seen in HCPS caused by SNV or New York virus [34, 227-231]. Hantavirus has also been described to infect squamous epithelium and capillary endothelium of the soft palate in Chinese patients with HFRS [226].

Regarding PUUV induced HFRS, reflecting the low case fatality rate, there is only one published report describing the distribution of viral antigen in various organs and tissues in the human body. In this single-patient case report of fatal PUUV infection, viral antigen was found in the spleen, kidney tubuli, and in the hypophysis' vascular endothelium and neuroendocrine cells. Additionally, PUUV RNA was detected (by RT-PCR) in samples from hypophysis, kidney, lung and spleen, while samples from brain, heart and liver were negative for viral RNA [104]. PUUV antigen has also been detected in endothelium in human salivary gland [232] and gastrointestinal tract [99, 233]. There are only few reports of hantavirus detections in samples from the airways. Investigating bronchoalveolar lavage, PUUV RNA was detected

in small cases series, whereas SNV RNA was not detected in bronchial secretions [123, 187].

A study of PUUV infected macaques similarly showed viral antigen in renal tubular epithelium, endothelium of spleen, liver and kidneys, along with staining in liver Kupfer cells [217]. In the lethal ANDV hamster model, viral antigen was detected in vascular endothelium of all internal organs, in the alveolar lining epithelium, and in cells of macrophage morphology [140]. In suckling mice infected with HTNV, viral antigen was found primarily in vascular endothelium in all organs and tissues [219].

In summary, hantaviruses have *in vivo* been shown able to infect a wide range of cells types in multiple organs. Cells reported most frequently infected were endothelial cells, widely considered the primary target cell for hantaviruses [5, 34, 211]. Although properties of vascular endothelial cells vary between organs, it is still unknown why different hantaviruses show relative preference for endothelium of specific organs, seemingly connected to the clinical manifestations related to the viruses [34]. Viral interactions with co-factors differently expressed by the endothelium in addition to integrins may be one potential explanation [234, 235].

Cell-mediated immune responses

Infiltrates of small and large mononuclear cells, some with appearance of immunoblasts, have consistently been reported in necropsy samples from patients with fatal hantavirus disease [34, 77, 121, 122, 134] as well as in kidney biopsies from survivors [236, 237]. The cellular response has been detected in all investigated organs and tissues, including the lungs, heart, kidneys, spleen, lymph nodes, liver, muscles, and pancreas, reflecting the widespread systemic infection. Interestingly, the magnitude of cell infiltrates were in SNV-related HCPS reported to correlate to the clinical presentation, meaning most inflammatory cells were detected in the lungs compared to the kidneys [34, 134]. In peripheral blood, expansion of leukocyte numbers (leukocytosis) is typically seen. In severe hantavirus disease, the leukocytosis may be extreme and described as a leukemoid reaction (leukocyte counts sometimes exceeding 100 x 10^9/L) [34, 87, 121]. Employing immunohistochemistry, mononuclear cell infiltrates were shown to consist mainly of a mixture of T cells, marked by a predominant expansion of CD8+ cytotoxic T cells (CTL) and cells of monocyte/macrophage lineage, along with occasional natural killer (NK) cells [34, 134, 236, 237]. B cells were only rarely detected in tissues, but have been reported to participate in the leukemoid reaction [34, 87, 121].

In blood, strong expansions of cytotoxic lymphocytes, CTLs and NK cells, have been reported both in HFRS and HCPS patients [178, 204, 205, 238-

241]. However, during early disease, transient lymphopenia including cytotoxic subsets has been described [16, 241-244], in similarity with many other acute viral infections [245, 246]. High proportions of detected CTLs were shown to be virus-specific and displaying evidence of proliferation and activation [178, 204, 205, 239, 240, 247, 248] suggesting an activated effector phenotype in these cells, furthermore supported by detection of granular cytotoxins granzyme B and perforin in almost all responding CTLs [239].

Regulatory T cells are important regulators of the immune system, functioning mainly by suppressing effector T cell responses to achieve balance between clearing an intruding pathogen and collateral damage of overly strong immune responses [249]. If the infecting microorganism manages to induce strong regulatory responses in the host it may help prolong pathogen survival and thereby lead to a chronic infection, as has been described in several human viral infections (e.g. herpes viruses and hepatitis B and C) [203, 249, 250], as well as in hantaviruses natural rodent hosts [55, 58]. On the contrary, if the regulatory response during infection is too weak, as has been hypothesized for human hantavirus infections (Figure 2), it may lead to pathogen clearing, however, at the cost of a potentially severe disease [85, 249, 250]. Accordingly, either reduced numbers or lack of regulatory T cell expansion have been demonstrated in blood in human hantavirus disease [239, 251, 252], as well as in infections with two other viruses causing disease with significant immunopathogenesis, namely West Nile fever and dengue hemorrhagic fever [253-255]. Regulatory T cell responses were also suppressed in the lethal ANDV hamster model for HCPS [140], giving further support that non-preferential expansion of regulatory T cells may be involved in hantavirus disease pathogenesis.

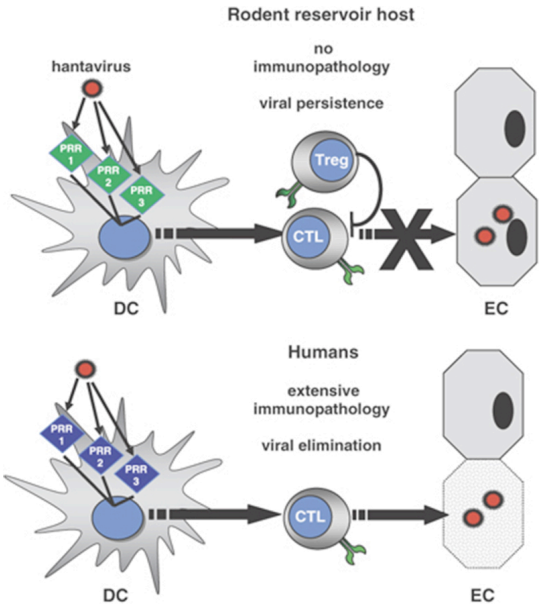

Figure 2. Postulated differences in regulation of hantavirus-specific immune responses in the reservoir hosts and in humans (Schönrich et al. 2008 [85]). In the natural host, the viral interactions with dendritic cells (DC) and pathogen recognition receptors (PRR) could lead to stimulation of regulatory T cells (Treg) to suppress virus-specific cytotoxic T cells (CTLs) and thereby mediate viral persistence and avoidance of immunopathology. In humans, PRR signaling in DCs could instead lead to a dominant antiviral CTL response attacking infected endothelial cells (EC) and causing immunopathology. Figure reprinted with kind permission from the publisher.

Increased production of proinflammatory cytokines (e.g. IL-1, IL-6, TNF-α) by monocytes/macrophages and lymphocytes are believed to be involved in development of many of the general symptoms related to hantavirus infections as well as vascular leakage, myocardial depression and hypotension [120, 138, 146, 179, 256, 257]. Higher numbers of cytokine producing cells have been detected in the lungs of patients with HCPS [179], compared to the kidneys, reflecting both clinical manifestations and distribution of virus. Of special interest, TNF-α, which is mainly released by activated macrophages but also from T cells and endothelial cells, has often been detected in blood early in hantavirus disease. Whether high circulating levels of TNF-α lead to severe disease have not been clearly shown as some studies have shown correlation to indicators for more severe disease while other studies have not [120, 145, 256, 258]. On the other hand,

polymorphism in TNF-α allele related to TNF-α hypersecretion phenotype have been associated with severe PUUV infections in Finland and Belgium [259-261], as well as developing HCPS when infected with Araraquara virus (ANDV related virus) in Brazil [262] and higher mortality risks in several studies of patients with severe sepsis [263].

Genesis of vascular leakage

It was proposed that capillary hyperpermeability, hallmark of hantavirus disease, could be the result of activated hantavirus-specific CTLs performing effector responses on targeted infected endothelial cells [69, 84, 85, 178]. This hypothesis finds support by the discovery of higher numbers of virus-specific CTLs in patients with severe outcome in SNV infection [204] and the discovery that SNV-specific CTLs from HCPS patients could induce vascular leakage in SNV infected endothelial cell monolayers [264]. Furthermore, CTL effector proteins (perforin and granzyme B) correlated to markers for apoptosis in serum from patients with PUUV infection, suggesting CTL-induced apoptosis of infected endothelial cells could contribute to vascular leakage [265]. On the other hand, necropsy and biopsy studies have not been able to show the presence of injured endothelium [34, 77, 231]. This suggests that the vascular leakage could instead be related to endothelial dysfunction, perhaps caused by local cytokine release from activated cytotoxic lymphocytes and macrophages, rather than widespread damage caused by cytolysis [84, 138, 179]. Further evidence for this perspective comes from *in vitro* studies showing hantavirus virus infection makes cells resistant to apoptosis induced by NK cells [63, 64], perhaps explaining the lack of evidence for endothelial apoptosis in heavily infected vascular beds [34]. However, increased numbers of circulating endothelial progenitor cells, with function to repair damaged endothelium, have been described in hantavirus disease [266], and may speculatively suggest that killed infected or noninfected bystander endothelial cells are seamlessly being replaced.

Other mechanisms suggested to contribute to vascular leakage in hantavirus infection have included direct viral effects by downregulation of inter-endothelial cell junctions [212], activation of the bradykinin system [79], prolonged endothelial sensitivity to TNF-α [267], virus or hypoxia induction of vascular endothelial growth factor [268], extensive platelet adhesion to endothelia [211, 213], and degradation of endothelial glycocalyx serving as a primary barrier of the endothelium [269].

Although CTLs have been attributed negative effects in hantavirus disease, they are essential in clearance of viral infections, as has been shown in an immunodeficient animal model for HTNV infection [218]. Furthermore, high numbers of virus-specific and interferon-γ secreting CTLs appeared to protect against severe disease in human HTNV infection [247]. The picture

became somewhat more opaque when it was shown that hamsters challenged with ANDV or SNV developed lethal HCPS even after almost complete depletion of CTLs [270, 271], thus questioning the role of CTLs in hantavirus pathogenesis not just in the hamster model but more importantly in humans.

Disease severity

Investigating markers for important aspects of pathogenesis in relation to disease severity may help improve understanding of relative importance of different pathways and suggest mechanisms for generated pathology. Thereby, it could help in proposing appropriate targets and timing for treatment. Hantavirus disease severity has in studies been suggested attributed to the level of early viremia [189, 192, 220, 272], strong CTL [204, 205] and general cell-mediated immune response (leukocytosis) [27, 73, 76, 95, 96, 205], low specific IgG response [76, 186, 273], intense proinflammatory or low antiinflammatory cytokine responses [120, 145, 146, 256], low numbers of regulatory T cells [252], hosts' HLA haplotype or TNF-α allele polymorphisms [206, 207, 210, 261, 262, 274], and high infectious dose [82].

Treatment

The cornerstone of hantavirus disease treatment has always been supportive care as there is still no effective specific treatment available [5, 275]. Important aspects of supportive care include management of fluid balance, electrolytes, hypotension, organ perfusion, oxygenation, and to ameliorate patient discomfort (such as nausea and pain). First World War recommendations advocated doctors providing patients large volumes of warm fluids (4.5 L/24 h of milk, barley water, and lemonade) through the oliguric phase. This treatment was considered to be highly effective since polyuria soon followed, although consistently with peripheral edema and systemic hypertension preceding [1]. Since the 1950s, treatment recommendations have stressed the importance of maintaining fluid balance according to input and output to reduce morbidity and mortality [130, 156, 157, 276]. Cardiogenic shock and pulmonary failure require intensive care unit treatment. The goal should be to maintain organ perfusion and oxygenation and not to achieve normal blood pressure readings. Hypotension is initially treated with intravenous crystalloids or concentrated albumin, but fluid therapy may be of short value and show negative consequences by excessive edema [130, 156, 157, 276]. Vasoactive drugs are used as appropriate considering the hemodynamic situation. In HCPS patients, inotropic agents (e.g. dobutamine) have been recommended over

vasoconstrictors [124, 130]. Norepinephrine, mainly a vasoconstrictor, was shown to raise blood pressure in HFRS patients, but did so merely by increasing peripheral vascular resistance showing no positive effects on capillary leakage, organ function or mortality [152, 277]. Mechanical ventilation support, either by noninvasive or invasive (i.e. intubation) techniques may be necessary to maintain oxygenation in patients with respiratory failure due to pulmonary edema. Extracorporeal membrane oxygenation may improve survival in severe HCPS [123, 276, 278].

Given that hantavirus disease is believed to be largely immune-mediated, corticosteroids could be expected to be beneficial, as reported in single cases [171]. Corticosteroids have however failed to show any significant value in placebo-controlled double-blind trials as well as in series of open-label use [156, 279, 280]. Cyclophosphamide, a drug suppressing B and T cells, evaluated in Chinese HFRS patients showed limited value [281] (referred by [282]).

Intravenous ribavirin, having *in vitro* activity against hantaviruses, has been demonstrated first in a placebo-controlled double-blinded study and later in open trials with comparisons to historical controls to significantly reduce both morbidity (e.g. risk of oliguria, hemorrhagic manifestations and degree of kidney injury) and mortality in Chinese and Korean HFRS [283, 284]. Ribavirin has, however, failed to show any success in treatment initiated in cardiopulmonary phase of HCPS in the United States [285]. Side effects were reversible mild to moderate anemia and time dependent cardiac bradyarrythmia of uncertain relation [284].

Serum or plasma from convalescent immune donors may pose an attractive treatment, since severe disease has been linked to host inability to mount an appropriate B cell response and produce neutralizing antibodies [76, 186, 273]. Although immune serum was tried without success in small number of patients with HTNV-related HFRS patients [156], it did show a tendency towards reduced case fatality rate in HCPS caused by ANDV infection in a nonrandomized trial, and it was safe to use [286].

Antagonists of bradykinin (icantibant or deltibant), a mediator of vascular leakage and dilatation, have been used in small number of patients with hantavirus disease and has shown some potential, but more studies are needed [123, 175, 177].

Aims

The general aim in the work leading to this thesis was to improve knowledge of cardiopulmonary involvement and its causes in Puumala virus infection.

The specific aims were to

- Describe cardiopulmonary manifestations in Puumala virus infection.
- Investigate the pathogenetic mechanisms responsible for cardiopulmonary involvement in Puumala virus infection.
- Define the local immune response in the lungs of patients infected with Puumala virus.
- Determine the presence of Puumala virus in samples from the lungs.

Subjects and methods

Study subjects

All study subjects were recruited from the cohort of patients with verified PUUV infection (IgM and IgG positive) hospitalized at the Department of Infectious Diseases (University Hospital, Umeå, Sweden). In the study for paper I, we identified and included PUUV infected patients (n=3, 2 females and 1 male, age 63-73 years) from the outbreak in 2007 that were experiencing severe cardiopulmonary failure leading to the need of invasive mechanical ventilation in the intensive care unit.

The study for paper II included hospitalized patients (n=15, 6 females and 9 males; median age 49 years, range 22-66) investigated 1994 to 1998.

Patients recruited in studies for papers III (n=27, 18 females and 9 males; median age 54 years, range 19-82) and IV (n=17, 11 female and 6 males; median age 54 years, range 31-69) were consecutively enrolled 2008-2012. Subjects in paper IV were all apart from one patient a subgroup consisting of patients in study III who accepted bronchoscopy. Another 19 patients (7 females and 12 males) were hospitalized during the same timespan but were not included either due to short hospitalization (<2 days, n=7), lack of consent (n=8), or logistical reasons (n=4).

In the studies for paper II-IV, we recruited controls available in previously collected databases of healthy volunteers participating in studies at the Department of Public Health and Clinical Medicine (paper II and IV) and the Department of Surgical and Perioperative Sciences (paper III). Controls for study II (n=14) included 4 females and 10 males with a median age of 23 years (range 21-27 years). In study for paper III, we recruited controls (n=25, 17 females and 8 males; median age 56 years, range 42-78) that were matched for sex and age. Controls in study for paper IV (n=16, 10 females and 6 males; median age 62 years, range 50-71 years) were matched according to sex, age, and smoking habits.

Clinical data and disease severity

Clinical data, including reported symptoms, pulse oxymetry and arterial blood gas analyses, arterial blood pressure, body weight, fluid balance, treatments, and length of hospital stay were retrieved from the patients' medical records. Markers considered to represent more severe disease were all related to important features of hantavirus disease, such as vascular and organ dysfunction.

Bronchoscopy, biopsy sampling and bronchoalveolar lavage

Bronchoscopy allows for visual inspection of the airway, sampling of endobronchial mucosal biopsies and performance of bronchoalveolar lavage (BAL). Bronchoscopies were performed using a flexible video bronchoscope (Olympus BF IT200 [paper II] or BF IT160 [paper IV]; Tokyo, Japan) with patients in supine position. Thirty minutes prior to bronchoscopy, patients were given premedication with subcutaneous morphine-scopolamin or atropine, alternatively intravenous glykopyrron often in combination with oral midazolam. Topical anesthesia was achieved during the procedure by sprays of lidocaine. All bronchoscopies were performed when platelet counts were recovering and deemed sufficient (>100 x 10^9/L) to avoid bleeding complications. Bronchoalveolar lavage was retrieved from the right middle lung lobe by infusions of 3 x 60 mL aliquots of phosphate buffered saline (PBS; paper II) or 3 x 60 mL aliquots of 0.9 % saline solution (paper IV), that was gently sucked back and pooled in a container kept in ice water. Bronchoalveolar lavage method aims by the relatively large volumes to reach and sample the distal airways, especially reflecting the conditions in the alveoli. Endobronchial mucosal biopsies (4-6 per subject, paper II) were sampled from the main carina and main left bronchial divisions using fenestrated forceps (Olympus FB 21C; Tokyo, Japan), thereby allowing analyses of bronchial tissue.

Bronchoalveolar cell differential count

The chilled bronchoalveolar lavage fluid was filtered through a nylon filter and centrifuged. The cell pellets were resuspended in PBS, cells were counted and then diluted to a final concentration of 10^6 cell per mL used for flow cytometry. Differential cell count was performed on cytospin slides by manually counting 500 nonepithelial cells per slide, giving numbers of macrophages, neutrophils, lymphocytes, and eosinophils per mL of BAL fluid.

Flow cytometry

Flow cytometry allows for distinguishing between different cell types and, by use of flourochrome labeled monoclonal antibodies directed at cell proteins of interest, makes it possible to determine specific cell subsets. The flow cytometer uses a laser beam that hits cells in suspension that passes by in a single line at up to several thousand cells per second. It evaluates individual cells based on cell volume (forward scatter), inner complexity (side scatter), and emitted light, recorded by detectors. Lymphocyte subsets were accordingly determined in BAL fluid (paper II and IV) and in peripheral

blood (paper II) using flourochrome labeled monoclonal antibodies. Defined lymphocyte subsets were: T cells (CD3+), T helper cells (Th cells, CD3+CD4+), cytotoxic T cells (CTL; CD3+CD8+), natural killer cells (NK cells, CD3-CD16+CD56+), and B cells (CD19+). Regulatory T cells were determined as CD4+ T cells displaying high ('bright') expression of CD25 (IL-2 receptor α subunit) combined with low or absent expression of CD127 (IL-7 receptor α subunit) (CD3+CD4+CD25'bright'CD127'low/-') as proposed [287]. CD25, CD69, HLA-DR and NKG2D were used as markers for T cell activation. Flow cytometry was performed using a Becton Dickinson FACSscan (paper II) or a Becton Dickinson FACSCalibur flow cytometer (paper IV), collecting 10 000 total events (paper II) or up to 80 000 total events (paper IV) per sample. The lymphocyte population was gated by their physical characteristics, according to forward and side scatter profiles.

Tissue processing and immunohistochemistry

Tissue samples from lungs, heart, kidneys, brain, spleen, and liver obtained during necropsies (paper I) were embedded in paraffin and cut at 5µm. Immunostaining was performed using eosin and hematoxylin for standard morphology, or with monoclonal antibodies and immunoperoxidase technique to specifically detect PUUV nucleocapsid protein (antibody A1C5, Progen Biotechnik, Heidelberg, Germany), alveolar macrophages (CD68+), T cells (CD3+), Th cells (CD4+), and CTLs (CD8+). In addition, sections were stained for granzyme B and T cell restricted antigen-1 as markers for cytotoxic effector activity.

Bronchial biopsies sampled during bronchoscopy (paper II) were processed into glycol methacrylate (GMA) resin (Polyscience, Northampton, England) [288] followed by storage at -20°C until cutting and immunostaining. Embedding the biopsies in GMA resin preserves tissue and cell morphology better than paraffin and allows for cutting thin sections (1-2 µm). Biopsies were cut at 2 µm and then stained using monoclonal antibodies to detect T cells (CD3+), Th (CD4+), CTLs (CD8+), neutrophils (neutrophil elastase, NE+), and eosinophils (eosinophil cation protein, ECP+). In addition, endothelial cell adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and P-selectin, were studied as markers for endothelial activation. Biopsy reading was performed by one of the authors blinded to subject groups. Only areas with intact epithelium and submucosa were evaluated. The total length of epithelium and area of submucosa were calculated using computer assisted image analysis (Qwin, Leica Q500IW; Leica, Cambridge, UK). Results were expressed as number of positive cells per mm of epithelium and cells per mm^2 of submucosal area, or as proportion of submucosal endothelium

stained with specific adhesion molecules in relation to total number of vessels in biopsy revealed by staining with panendothelial monoclonal antibody (EN4).

Analyses of granzymes and selected cytokines

Granzymes are cytotoxic granular effector molecules of cytotoxic lymphocytes (CTLs and NK cells), released to induce apoptosis in targeted cells [289-291]. Interleukin-6 and TNF-α have in previous studies been associated with hantavirus pathogenesis and disease severity. Thus, we wanted to study concentrations of these cytotoxic molecules and proinflammatory cytokines in BAL fluid to evaluate important effector immune responses in the lungs in PUUV infection and their relation to other study parameters. In studies for paper IV, we used commercial enzyme-linked immunosorbent assay (ELISA) kits to analyze BAL fluid concentrations of granzyme A (Biovendor, Brno, Czech Republic), granzyme B (Abcam, Cambridge, MA, USA), IL-6 and TNF-α (R&D Systems, Abingdon, UK).

Quantitative RT-PCR

To determine the viral load in studies for paper I and IV, Puumala virus RNA was analyzed by quantitative real-time RT-PCR, as previously described (Evander 2007)[185]. In studies for paper IV, cDNA was generated using GoScript™ Reverse Transcription System (Promega Biotech, CA, USA). Furthermore, the generated cDNA was pretreated with HK™ UNG (Epicentre Technologies, Madison, WI) to avoid any contaminating RNA that could be present in the BAL fluid.

Lung function

Using computerized Jaeger equipment (Würzburg, Germany), lung function was determined by spirometry (flows and volumes) and measurements of diffusing capacity of the lung for carbon monoxide (DLCO) and total lung capacity. Investigations were performed during hospitalization in the acute phase of disease and repeated 2 months (paper II) and 3 months (paper III) after disease onset.

High-resolution computed tomography

To investigate potential thoracic abnormalities related to PUUV infection, high-resolution computed tomography (HRCT) studies were performed using a 64-slice LightSpeed VCT scanner (GE Healthcare, Milwaukee, WI, USA). Patients were investigated by HRCT in the acute phase of disease and

repeated as appropriate either by HRCT or standard chest X-ray at 3 months follow-up if abnormalies were present in the initial HRCT examination.

Cardiac function

In addition to resting ECG, echocardiography was used to evaluate cardiac function in acute phase and at 3 months follow-up according to current guidelines [292-295].

Echocardiographic parameters included measured dimensions and calculated volumes of left ventricle and atrium during systole and diastole that were also used for calculating left ventricular ejection fraction. Doppler recordings of blood flows were used to evaluate the filling pattern of the left ventricle, measure left ventricular stroke volume allowing calculation of cardiac output (heart rate x stroke volume), and assess diastolic function partly by isovolumic relaxation time (IVRT). Further investigations of right heart function included Doppler measurements of peak tricuspid regurgitation pressure gradient to allow estimations of peak systolic pulmonary artery pressure and secondary calculations of pulmonary vascular resistance [296], further assessed by measurements of pulmonary artery acceleration time [297]. Determined tricuspid annular plane systolic excursion (TAPSE) was used as an indirect measure for right ventricular ejection fraction [298], while right ventricular IVRT was measured to assess the diastolic function of the right ventricle [299]. In addition to these standard two dimensional and Doppler measurements, speckle tracking echocardiography technique was employed. “Speckles” are grain-like artifacts of echocardiography that move together with the tissue. Using specialized software, these grains can be used for continuous tracking and thereby providing detailed information on myocardial deformation and contractility [300, 301].

Cardiac biomarkers

Troponins (C, T, I) are enzyme components of myocardial sarcomere thin filaments that are involved in generating muscle contraction. They are released in blood when cardiomyocytes are damaged and therefore commonly used when diagnosing myocardial infarction or myocarditis. B-type natriuretic peptide (BNP) and its inactive by-product N-terminal-proBNP (NT-proBNP) are typically released in blood in situations involving excessive myocardial stretch and used to evaluate heart failure [302], but have also been shown to be induced by proinflammatory cytokines that also exhibit cardiodepressing effects [143, 303, 304]. Natriuretic peptides have a cardioprotective role by several mechanisms, including natriuresis, diuresis, vasodilatation, and suppression of renin-angiotensin-aldosterone system

[303, 305]. We investigated plasma concentrations of troponin T, using high-sensitivity assay, and NT-proBNP, both analyzed at the local accredited hospital laboratory, as biomarkers to detect myocardial cell damage and cardiac dysfunction in PUUV infection.

Statistical analyses

Nonparametric tests were consistently used in the studies for this thesis and data were expressed as median (25^{th}-75^{th} percentile), unless otherwise stated. The reason for this was that sample sizes were small and data were often not normally distributed. Mann-Whitney U test was used for comparison between groups (e.g. patients versus controls), while Wilcoxon signed-ranks test was used for paired within-group observations (e.g. acute phase versus follow-up). Spearman's ranked correlations coefficient was used for correlation analysis and Fisher's exact test was used to compare categorical data. All tests were two-tailed and a p-value <0.05 was considered statistically significant.

Main results and discussion

Paper I – Severe Puumala virus infection

In this small case study, we described three patients with acute PUUV infection leading to need of intensive care treatment including invasive mechanical ventilation along with vasopressor and inotropic support for acute cardiopulmonary failure. After 1-4 days of a febrile prodrome also including dyspnea and dry cough, patients rapidly deteriorated displaying circulatory shock, noncardiogenic pulmonary edema and large thoracic effusions (Figure 1, paper I), in similarity to patients with HCPS induced by American hantaviruses [27, 75]. Two patients died and one survived. Patients showed varying degrees of renal impairment ranging from mild in the two fatal cases to more severe, in terms of anuria and dialysis, in the survivor. Other important aspects of disease were somnolence (two out of three patients), implying involvement of central nervous system, and disseminated intravascular coagulation (all three patients) in terms of deep thrombocytopenia, prolonged activated partial thromboplastin time, and elevated prothrombin complex international normalized ratio. Clinical manifestations of coagulopathy were petechiae and thrombosis. Pulmonary hypertension was noted in one of two investigated patients (estimated systolic pulmonary artery pressure >65 mmHg), in parallel to patients with HCPS or ARDS [124, 131-133]. Autopsies in the two fatal cases revealed edematous lungs containing high number of alveolar macrophages and parenchymal mononuclear cell infiltrates consisting of T cells, whereof a vast majority was of the CD8+ subset with expression of cytotoxic granular proteins granzyme B and TIA-1 in many mononuclear cells. This indicated the presence of a predominantly cytotoxic effector response in the lungs, further supported by markedly high serum LDH levels (maximum 8.7-30.8, reference <3.4 μkat/L) also described in HCPS [27, 74, 75]. One patient's heart contained focal massive infiltrates of neutrophils and macrophages, indicating myocarditis, similarly described in ANDV infection related HCPS [138]. Notably, kidneys contained no prominent inflammatory cell infiltrates showing that, in parallel to the premortal presentation, renal involvement was not an important clinical feature in these patients. Puumala virus RNA was detected by RT-PCR in organ samples from both fatal cases. Puumala virus antigen could only be visualized in one patient and was found mainly in the vascular endothelium and, to lesser extent, in mononuclear cells in all investigated organs, as previously reported concerning infections with other hantaviruses [34, 224]. Puumala virus RNA could be detected in serum sampled during incubation period three weeks prior to hospitalization, as reported in ANDV infection [43].

Hantavirus disease caused by PUUV varies in severity from mild to fatal. Why these three patients developed severe disease was not established but could perhaps be due to inability to mount neutralizing humoral responses [76, 186, 273], as indicated by delayed IgM and IgG in these patients. Other alternatives, not possible to support by available data, could be related to host factors or high infectious dose, shown in human or animal studies to be associated with severe disease [82, 206, 207]. Severe cardiopulmonary distress has recently been described as frequently occurring among patients with critical HFRS in China [91] showing many similarities to the cases presented here. This indicates that although acute renal failure is the most frequent and prominent organ manifestation in HFRS, the clinician must be aware of cardiopulmonary failure, sometimes even in absence of obvious renal involvement, as a clinical presentation of hantavirus disease even in the Old World.

Paper II – Immune responses in blood and lungs

Immune responses in the lungs and peripheral blood were studied in 15 patients with PUUV infection and compared to 14 control subjects. As in previous reports [95, 96, 98], respiratory involvement was frequently seen, indicated by symptoms (7 out of 15 patients), hypoxemia by arterial blood gas (7 out of 12 patients), infiltrates on chest X-ray (4 out of 15 patients), and need of oxygen treatment (2 out of 15 patients). Further demonstrating impaired gas exchange and thereby involvement of the alveoli, patients showed reduced DLCO during acute infection that significantly improved at follow-up after 2 months (75% [63%-95%] versus 94% [88%-102%]; median [25^{th}-75^{th} percentiles]; p=0.007).

Patients' bronchial mucosal biopsies contained, compared to controls, higher numbers of CD8+ T cells in submucosa (p=0.001) and epithelium (p<0.001) (illustrated in Figure 3) and higher numbers of CD4+ T cells in submucosa (p=0.001). The submucosal vascular endothelium showed evidence of being activated by the infection in terms of higher expression of adhesion molecule VCAM-1 in patients, compared to controls (p<0.001).

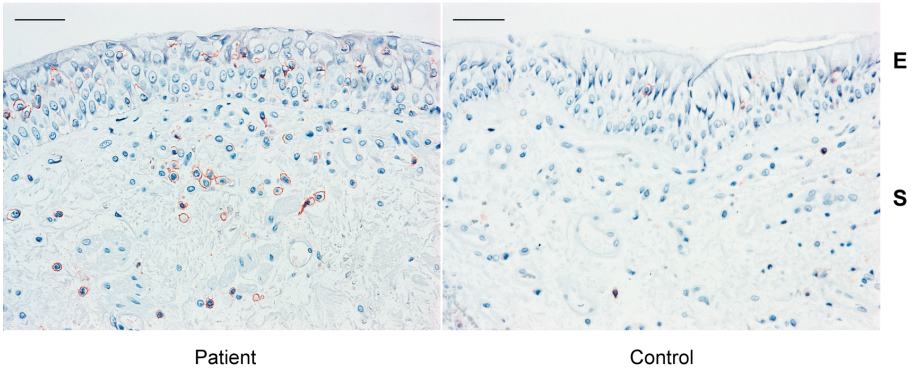

Figure 3. Endobronchial biopsies displaying high numbers of CD8+ T cells in epithelial (E) and submucosal (S) regions of the bronchial mucosa in hantavirus infected patient, compared to the uninfected healthy control. Positive cells show red ring staining pattern and the bars represent 50 μm.

Flow cytometry of bronchoalveolar lymphocytes showed proportional expansions of NK cells ($p<0.001$), T cells ($p=0.011$), CTLs ($p=0.040$), in patients, compared to controls, while bronchoalveolar B cell and CD4+ T cell proportions were similar between the groups. The pulmonary T cell population showed evidence of strong activation during hantavirus infection, indicated by approximately 50-fold higher expression of HLA-DR ($p<0.001$) and 5-fold higher expression of CD25 ($p<0.001$) in patients, compared to controls.

In blood, a slightly different lymphocyte response was detected, in terms of higher proportions of B cells ($p=0.042$) in patients, compared to healthy controls, while proportions of T cells and CD8+ T cells were similar comparing the groups ($p=0.549$ and $p=0.420$, respectively). On the other hand, detected T cells in blood showed 10-fold higher level of activation, in terms of HLA-DR and CD25 expression, in patients ($p<0.001$ for both), compared to controls. These observations could be related to differences in investigated compartments (i.e. the lungs versus blood) or to previously described temporal changes in blood lymphocytes during disease course. Transient early lymphopenia has been shown in many virus infections, including hemorrhagic fevers such as those caused by dengue and hantavirus [16, 241-243, 245]. The phenomenon has been proposed related to lymphocyte homing to lymphoid and other tissue or to increased endothelial stickiness [306]. Speculatively, apparent lack of T cell expansion in blood in the current report, as previously [241, 244], could be related to these cells adhering to activated endothelium or extravasating into infected tissues.

The accumulated results showed that PUUV infection led to pulmonary manifestations characterized by impaired gas exchange. This indicates alveolar involvement with an activated cytotoxic immune response found in

the same compartment. Reduced gas exchange, suggestive of increased distance for diffusion, could in hantavirus infection be related to pathology arising on either the endothelial or alveolar side of the blood-air barrier, or combined. Strong alveolar immune responses and related inflammation could, such as suggested here, contribute to reduced pulmonary function in hantavirus disease. The study could not demonstrate any correlation between pulmonary manifestations, including DLCO results or level of hypoxia, and the immune response in the lungs in terms of numbers of cells in tissue or proportions of lymphocyte subsets in bronchoalveolar space.

Comparing patients' blood lymphocyte populations in acute phase and at follow-up after 2 months, most subsets showed contraction, excluding NK cells that were surprisingly expanding. However, except for proportional activation level on total T cells, the comparisons did not reach statistical significance. Compared to the healthy controls, proportions of CD25 and HLA-DR activated T cells and NK cells in blood were still significantly higher ($p=0.004$, $p=0.001$, and $p=0.020$, respectively) in convalescent phase patients and indicate long lasting immune activation in hantavirus infection, similarly shown in another study [241].

Paper III – Cardiopulmonary involvement

Cardiopulmonary involvement was studied in 27 patients with PUUV infection and compared to 25 controls regarding echocardiography data. The results showed that PUUV infection frequently led to pathological changes in the thoracic compartment, most often as signs of increased vascular leakage in terms of effusions or pulmonary edema (Figure 1, paper III) detected in 11 out of 24 investigated patients (46%). Echocardiography could not show any evidence of intravascular volume overload or overt heart failure. Therefore, we concluded that thoracic effusions and pulmonary edema were most likely related to capillary hyperpermeability, in similarity to patients with HCPS [27, 75, 124].

Eighteen patients (67%) reported respiratory symptoms, in terms of dyspnea (n=14) and/or dry cough (n=10). Abnormal chest HRCT was associated with a stronger inflammatory response in blood (max CRP, $p<0.05$; max leukocyte count, $p=0.053$), an impaired alveolar gas exchange indicated by lower DLCO ($p<0.05$), and need of supplemental oxygen treatment ($p<0.05$) given to 33% (n=9) of patients due to desaturation and/or dyspnea. Patients requiring oxygen showed, beside lower DLCO ($p<0.05$), indications of more intense inflammatory response (max leukocyte count, $p<0.01$) and vascular leakage (min albumin, $p<0.01$), along with impaired organ function indicated by markers for renal impairment (max creatinine, $p<0.05$) and cardiac dysfunction (max NT-proBNP, $p<0.01$; max troponin T, $p<0.05$),

compared to patients not needing oxygen treatment. This suggests that patients failing in one organ system (i.e. the lungs) show dysfunction also in other organs and thereby points to the systemic nature of hantavirus infections, perhaps with capillary hyperpermeability and inflammation as the central mechanisms.

Main results from echocardiographic examinations revealed that hantavirus infected patients, compared to controls, displayed evidence of right ventricle involvement due to increased afterload, in terms of higher estimated pulmonary vascular resistance ($p<0.05$) shorter pulmonary artery acceleration time ($p<0.05$), and higher systolic pulmonary artery pressure ($p<0.01$) indicating mild to moderate pulmonary hypertension in patients (patient median 36mm Hg, range 22-52; controls median 26 mmHg, range 21-39). In addition, compared to controls, patients showed prolonged biventricular isovolumic relaxation times (left ventricle, $p<0.05$; right ventricle, $p=0.057$) and lower left ventricular ejection fraction ($p<0.01$), although these were rarely subnormal in absolute terms and thereby suggestive of discrete diastolic and systolic dysfunction. Similar results regarding ejection fraction have previously been shown for patients surviving PUUV or SNV related HCPS, whereas fatal HCPS cases displayed markedly reduced ejections fractions [124, 180]. In addition, impaired left atrial contractile function was suggested by reduced atrial strain rate in acute infection, compared to the 3 months follow-up and the healthy controls ($p<0.01$ for both), by speckle tracking echocardiography.

Electrocardiography performed in the acute phase (n=27) revealed inversions of T-waves in seven patients (26%) and atrial fibrillation in two cases. The results parallel those reported in larger ECG studies in HFRS [16, 101, 180, 182]. At follow-up after 3 months, most pathological changes had normalized except in two patients, whereof one still displayed atrial fibrillation and the other showed remaining changes in the T-waves.

Patients receiving oxygen treatment, compared to those not needing supplemental oxygen, displayed shorter TAPSE ($p<0.05$) along with higher estimated pulmonary vascular resistance ($p<0.05$) and systolic pulmonary artery pressure ($p<0.01$). This observation suggests a relation between severity of pulmonary manifestations in PUUV infection and right heart systolic function, in terms of increased afterload and impaired right ventricle contractility, as in HCPS and ARDS [124, 131-133]. Furthermore, high NT-proBNP concentrations were associated with presence of pulmonary edema ($p<0.01$), low pulmonary DLCO ($p<0.05$), and high vascular resistance ($p<0.05$) and could indicate increased myocardial load and distress secondary to changes within the pulmonary vasculature. Interesting in this aspect is the reports of overt right heart failure in patients with severe

pulmonary edema during HTNV infection [307] or lethal H1N1 influenza A ARDS [133], known as pulmonary heart failure or *cor pulmonale,* though none of the patients in the present cohort were near as sick.

In the complex situation of an acute disseminated infection, such as by a hantavirus, it is difficult to separate primary and secondary effects. Furthermore, by the descriptive nature of the study, conclusions concerning causality cannot be made with certainty. The cardiac biomarker NT-proBNP is used to diagnose and evaluate heart failure. Renal failure and proinflammatory cytokines have been shown to increase the concentration of NT-proBNP in plasma [303, 304, 308, 309] and in the current report, the concentration of NT-proBNP showed a temporal peak in the late oliguric phase when renal impairment and inflammatory responses are also strong. Therefore, it is possible that the detected association between the pulmonary involvement and elevated concentrations of NT-proBNP was instead caused by renal failure or inflammatory response. However, patients did not display evidence of fluid overload and concentrations of NT-proBNP were in most patients markedly elevated already on admission and prior to renal impairment. NT-proBNP is cleared from plasma in tissues with large vascular beds, such as kidneys, muscles and liver, but the mechanism of clearance is still unknown. Although NT-proBNP concentration have been shown to correlate to markers for renal failure, the importance of kidney function in elimination of NT-proBNP is controversial [303, 310-312]. Furthermore, NT-proBNP was elevated in other infections in absence of kidney failure [304, 308, 313-316]. We believe that elevated NT-proBNP in hantavirus infection is an indicator of myocardial distress, be it secondary to the inflammatory response, renal failure, pulmonary involvement, or all of those.

Transient systemic hypotension (arterial systolic pressure ≤90 mmHg) was seen in one-third of the studied patients. Cardiac outputs were not increased despite the acute infection, when compared to controls or 3 months follow-up, and were significantly lower in patients with pulmonary edema ($p<0.05$). Whether these observations together with the finding of slightly reduced ejection fraction could reflect mild myocardial depression in patients with PUUV infection, as suggested in more severe HCPS and probably also in HTNV associated HFRS [91, 124, 138, 152, 181], is difficult to speculate upon. However, strain rate analysis did not, at least on group level, show any evidence of reduced left ventricle contractility during acute PUUV infection. The current patient cohort consisted of individuals with ranging severity, in our opinion, being typical representatives of the common spectrum of disease manifestations of PUUV cases at our clinic. It is possible more evident myocardial depression could have been revealed in severe cases, had

patient population been larger allowing stratification according to any definition of disease severity.

At 3 months follow-up (n=26), 13 patients (50%) reported impaired general condition, most often in terms of fatigue, tiredness, or exertional dyspnea, while the rest felt fully recovered. Reexamination of DLCO (n=24) showed significant improvements, compared to the acute phase ($p<0.001$). However, patients reporting impaired general condition displayed lower DLCO ($p<0.05$) as well as higher estimated pulmonary vascular resistance ($p<0.05$) and reduced TAPSE ($p=0.052$), compared to those who felt fully recovered. Furthermore, of the nine patients (38%) that displayed subnormal DLCO (<80% of predicted value) even after 3 months convalescence, eight were in the group that reported impaired general condition. Combined, this suggests remaining pulmonary manifestations may be responsible for the commonly reported long lasting fatigue after hantavirus infection, as similarly described in HCPS [317].

Paper IV – Viral load and immune responses in the lungs

In studies for paper IV, we investigated bronchoalveolar lavage fluid sampled from 17 patients with PUUV infection and 16 uninfected healthy controls. Of the 17 patients, that displayed typical clinical presentation for PUUV infection, about one-third (n=5) received oxygen treatment due to poor oxygenation and/or dyspnea and one-third showed transient hypotension (n=6, systolic blood pressure ≤90mmHg).

Bronchoalveolar lavage cell analyses revealed higher numbers of lymphocytes ($p=0.001$) in patients with PUUV infection, compared to controls. By flow cytometry, an inversed CD4/CD8 ratio was found in patients (Figure 1, paper IV). This was due to a strong proportional expansion of CD8+ CTL subset ($p<0.001$), displaying higher proportional expression of activation markers HLA-DR, CD25, and NKG2D ($p<0.001$, $p=0.010$, and $p=0.040$, respectively) in patients, compared to controls (Figure 1, paper IV). The bronchoalveolar lymphocyte expansion was in absolute numbers further defined by higher numbers of NK cells ($p=0.034$), CTLs ($p<0.001$), as well as CTLs expressing CD25 ($p<0.001$), CD69 ($p=0.046$), HLA-DR ($p<0.001$), and NKG2D ($p=0.001$) in patients, compared to controls. Together this indicated strong activation in the expanded pulmonary CTL population [318], in similarity with previous results in blood [239, 240]. Bronchoalveolar lavage fluid concentrations of cytotoxic lymphocyte granular effector proteins granzyme A and B were markedly higher in patients ($p<0.001$ for both), compared to controls. Additionally, the levels of the two cytotoxins showed significant correlation

to the numbers of activated CTLs in patients showing that the cytotoxic activity was proportional to the CTL response.

Puumala virus RNA was detected in BAL cells in 15 out of 17 patients. High number of bronchoalveolar CTLs, high concentration of granzymes, low viral load, and high serum LDH were all significantly interrelated, implying that CTLs could be involved in killing of infected cells within the bronchoalveolar compartment, thereby reducing viral load. However, what cells were infected was not determined. Considering results from other studies, it is likely these cells were mononuclear immune cells or respiratory epithelial cells, previously shown to be infected with hantavirus *in vivo* [34, 127, 176, 196, 221, 227].

The numbers of bronchoalveolar lymphocytes (p=0.015), T cells (p=0.020), CTLs (p=0.036), and NKG2D+ CTLs (p=0.020) were higher in oxygen-treated patients, compared to those not needing oxygen. Although not proving causality, these results suggest that strong cytotoxic pulmonary immune responses in PUUV infection may lead to impaired alveolar gas exchange and hence need of supplemental oxygen. Furthermore, high numbers of bronchoalveolar CTLs was associated with hypotension (nadir systolic blood pressure, r=-0.567, p=0.022), as well as blood surrogate markers indicating renal failure (maximum creatinine, r=0.54, p=0.030), cardiac dysfunction (maximum NT-proBNP, r=0.674, p=0.004; and maximum troponin T, r=0.406, p=0.023), cell damage (maximum LDH, r=0.56, p=0.025), and vascular leakage (minimum albumin, r=-0.50, P=0.050), likely reflecting the systemic nature of hantavirus infections. The relationship between intense lung immune responses and cardiac dysfunction markers could illustrate cardiac distress secondary to increased pulmonary involvement, similarly shown in paper III.

Lastly, pulmonary regulatory T cell population was not expanded and regulatory T cells were instead found to be fewer in patients (p=0.004), compared to controls. This finding corroborates previous results in blood [239, 251, 252] and may be seen as further evidence that insufficient regulatory T cell responses could contribute to hantavirus disease pathogenesis by allowing vigorous proinflammatory immune responses.

Concluding remarks

In the presented studies, we aimed to increase understanding of cardiopulmonary manifestations, their clinical relevance, and underlying pathogenetic mechanisms in hantavirus disease caused by PUUV. Varying degree of heart and lung involvement was detected in almost all patients, ranging from mild subclinical impairments to full-blown cardiopulmonary failure leading to fatal outcome.

The pulmonary involvement

Pulmonary dysfunction was best described by reduced lung gas diffusing capacity and hence need of oxygen treatment due to poor oxygenation. As in HCPS, the combined results strongly suggest pulmonary vascular hyperpermeability in the lungs being mainly responsible for detected lung effusions and impaired alveolar gas exchange, thus corroborating conclusions in previous reports [90, 95, 97, 98]. Even if no support for intravascular fluid overload could be noticed in the current patient cohort, it is reasonable to believe that fluid overload combined with a dysfunctional and leaking endothelium could aggravate the situation. Patients were deliberately kept at neutral fluid balance by restrictions of intake to prevent this and to improve clinical care. The results further indicated that strong CTL responses within the airway lumen could contribute to impaired pulmonary gas exchange, thereby suggesting that multiple mechanisms, on both sides of the blood-air barrier, may be involved. The pulmonary manifestations were long lasting and a probable cause of the commonly reported fatigue after acute hantavirus infection.

Cytotoxic lymphocyte response in the lungs

Only few studies have investigated whether strong cytotoxic lymphocyte responses could contribute to pathogenesis of hantavirus disease and results appear contradictory, as CTLs have been described as either harmful [204, 205], protecting [247], or irrelevant [270, 271]. However, different studies have used different methods and the question of what roles CTLs play in hantavirus disease have still not been solved. In the work presented in the current thesis, the pulmonary immune response could best be defined by a predominant expansion of activated CTLs performing cytotoxic activity within the bronchoalveolar compartment. The stronger the pulmonary CTL response was, the more severely ill the patients appeared. Interestingly, this observation included not just the local lung involvement, as commented on above, but also the severity of manifestations in other organs. The most straightforward explanation is likely that the pulmonary immune response

reflects similar immune responses present elsewhere in the body, thereby being indirectly correlated to dysfunction in other organs. The results presented in this thesis support the hypothesis that intense CTL responses, perhaps mediated by an insufficient regulatory T cell response, are important contributors to hantavirus pathogenesis [84, 85, 178, 197], as similarly shown in infections with other noncytopathogenic viruses [203, 253-255]. The observed inverse relation between activated CTLs and the local airway viral load suggests that CTLs are involved in the clearing of hantavirus infection, as could be expected considering CTL's function in the immune system. However, hantaviruses have been demonstrated to possess several immune evasive strategies and mechanisms of hantavirus clearance are not fully understood.

Presence of Puumala virus in the lungs

Viral replication within the airway is a requirement for airborne transmission between humans, as have been suggested occurring in ANDV infections in South America. We detected PUUV RNA in airway samples from almost all patients but whether this represents material from infectious virus has not been evaluated. However, the observation that viral RNA was rarely detected in the cell free portion could indicate that neutralizing humoral responses were present, as similarly shown in saliva [232] and in blood [76, 120, 220]. Combined with airway innate antiviral proteins [195, 319], this could likely explain the absence of described interhuman transmission for PUUV.

The cardiac involvement

The main cardiac manifestations were detected in the right heart and showed association with the magnitude of pulmonary involvement, as in ARDS and HCPS patients [124, 131-133]. The effects on right ventricle function were long lasting and, in parallel with the impaired pulmonary function, had not resolved fully 3 months after the acute infection. During the first month following HFRS disease onset, the risk for an acute myocardial infarction is strongly increased (up to 6-fold rise) [117]. Even if the most probable cause for infarctions after hantavirus infection is coagulopathy related to endothelial and platelet dysfunction, it is possible that increased myocardial stress during and directly after acute PUUV infection could contribute to a myocardial ischemic event.

Clinical implications and future perspectives

Definite clinical implications are hard to draw from descriptive studies. Some suggestions could be made however. Firstly, hantaviruses should be included in the differential diagnosis for febrile patients with acute cardiopulmonary distress in hantavirus endemic regions worldwide, especially in presence of thrombocytopenia. Secondly, patients relating long lasting fatigue in hantavirus convalescence may be evaluated by determination of lung function, including gas diffusing capacity.

So, should patients with hantavirus disease receive anti-CD8 therapy? Puumala virus infection causes a mild and self-limiting disease in almost all patients making drastic therapies with potentially severe side effects unattractive. Also, results from animal studies [270, 271] suggest a complex pathogenesis also in humans. There is, however, an urgent need of effective treatment especially in the more severe hantavirus infections.

Further studies to evaluate cardiopulmonary involvement in hantavirus disease could focus on cardiac arrhythmias and whether hantavirus infection causes any long-term effects on heart and lung function.

Conclusions

- Cardiopulmonary involvement of varying severity was present in almost all investigated patients with Puumala virus infection.

- Pulmonary dysfunction, in terms of variably reduced gas exchange, is a general feature of Puumala virus infection and was associated with long lasting fatigue, commonly reported during hantavirus convalescence.

- Cardiac manifestations were mainly evident in the right heart and secondary to changes within the lungs with findings of increased pulmonary vascular resistance, mild to moderate pulmonary hypertension and right ventricular dysfunction in patients with more pronounced lung involvement.

- The pulmonary immune response in Puumala virus infection was best described as expansion of highly activated CD8+ cytotoxic T cells. An intense immune response was associated with more severe disease.

- Puumala virus RNA was detected in bronchoalveolar lavage in almost all patients and the viral load was inversely correlated with the magnitude of the cytotoxic immune response. Viral protein was detected within the capillary endothelium of internal organs, including the heart and lungs.

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Det har varit många människor involverade i de kliniska studierna som leder fram till den här avhandlingen. Innan allt annat skulle jag vilja rikta ett särskilt tack till alla patienter som deltagit i studierna. Trots hög feber och alla andra vedermödor sorkfebern för med sig har ni hjälpt till att öka kunskapen om sjukdomen till gagn för andra.

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