Serum cytokine alterations associated with age of patients with Nephropathia Epidemica

Venera Shakirova[1], Ilseyar Khaertynova[1], Maria Markelova[2], Rachael Tarlinton[3], Jerzy Behnke[4], Ekaterina Martynova[2], Ekaterina Granina[2], Albert Rizvanov[2], Svetlana Khaiboullina[2]

[1]Department of Infectious Diseases, Kazan State Medical Academy, Kazan, Tatarstan, 420012, Russia.

[2]Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, 420008, Russia

[3]School of Veterinary Medicine and Science, University of Nottingham, Loughborough, LE12 9RH

[4]School of Life Sciences, University of Nottingham, University Campus, Nottingham, NG7 2RD

vene-shakirova@yandex.ru, i.khaertynova@gmail.com, mimarkelova@gmail.com, Rachael.Tarlinton@nottingham.ac.uk, Jerzy.Behnke@nottingham.ac.uk, ignietferro.venivedivici@gmail.com, kathryn.cherenkova@gmail.com, rizvanov@gmail.com, sv.khaiboullina@gmail.com

Corresponding Author

Dr. Ekaterina Martynova

Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Tatarstan 420008, Russia

E-mail: ignietferro.venivedivici@gmail.com

Dr. Svetlana Khaiboullina, MD, PhD,

Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Tatarstan 420008, Russia Email: sv.khaiboullina@gmail.com

Abstract

Nephropathia Epidemica (NE) is a zoonotic disease caused by Hantaviruses transmitted from rodents, endemic in the Republic of Tatarstan, Russia. The disease presents clinically with mild, moderate, and severe forms, and time dependent febrile, oliguric, and polyuric stages of the disease are also recognized. The patient’s cytokine responses have been suggested to play a central role in disease pathogenesis; however, little is known about the different patterns of cytokine expression in NE in cohorts of different age and sex.

Serum samples and clinical records were collected from 139 patients and 57 controls (healthy donors) and were used to analyze 48 analytes with the Bio-Plex multiplex magnetic bead-based antibody detection kits. Principal component analysis of 137 patient and 55 controls (for which there was full data) identified two components that individually accounted for >15% of the total variance in results and together for 38% of the total variance. PC1 represented a pro-inflammatory TH17/TH2 cell antiviral cytokine profile, and PC2 a more antiviral cytokine profile with patients tending to display one or the other of these.

Severity of disease and stage of illness did not show any correlation with PC1 profiles however, significant differences were seen in patients with high PC1 profiles vs lower for a number of individual clinical parameters: High PC1 patients showed a reduced number of febrile days, but higher maximum urine output, higher creatinine levels and lower platelet levels.

Overall, the results of this study point towards a stronger pro-inflammatory profile occurring in younger NE patients, this being associated with markers of acute kidney injury and low levels of high density cholesterol. This is consistent with previous work indicating that the pathology of NE is immune driven, with an inflammatory immune response being associated with disease and that this immune response is more extreme in younger patients.

Key words: Nephropathia Epidemica, serum, cytokine, hantaviruses, age

Introduction

Nephropathia epidemica (NE) is a mild form of hemorrhagic fever with renal syndrome (HFRS), a febrile zoonotic disease characterized by hemorrhages and renal pathology [1]. The disease has an acute onset with fever, headache, nausea, vomiting, hematuria and back pain [2-4] . Laboratory findings typically include thrombocytopenia, leukocytosis, decreased CD4:CD8 ratio, increased B lymphocytes counts and increased serum creatinine levels [4-9]. Acute kidney

injury is the major pathological finding and described in all cases. In severe cases, kidney failure can develop [10]. NE presents in three forms: mild, moderate and severe [11, 12]. Each form of the disease progression includes febrile, oliguric and polyuric periods, followed by convalescence. The severe form of NE is characterized by headache, vomiting, high fever (over 39.5°C) and acute kidney injury. The most prominent clinical features of this form of NE are hemorrhagic symptoms including petechial, nasal and internal bleeding [11-13]. The moderate form of the disease has similar symptoms but is more subtle. The mild form often remains undiagnosed. Symptoms are subtle including mild headache and fever (up to 38^{0}C), with the hemorrhagic syndrome restricted to small petechia on mucosa and skin. [14, 15].

NE is endemic in the republic of Tatarstan, Russia [16]. We have previously demonstrated that Puumala orthohantavirus (PUUV) is the primary cause of NE in Tatarstan [17]. It is believed that endothelial cells are the primary targets of PUUV, where the virus can replicate without a cytopathic effect [18]. This is supported by the lack of tissue damage commonly found in postmortem specimens [19]. Therefore, immune mechanisms have been suggested to play a key role in the pathogenesis of NE. We have previously shown activation of proinflammatory cytokines in the serum of NE patients [20], where the severity of the disease was associated with high levels of circulating TNF-α and IL-1β. We have also shown that the mild form of NE is characterized by increased serum levels of IFNγ and IL-12 [21]. Our data corroborate the findings of several other groups demonstrating cytokine production by infiltrating immune cells in the kidneys rather than the kidneys themselves. Based on a large body of data, it is generally considered that the clinical symptoms of NE are the result of a "cytokine storm" in response to the virus [22, 23].

There are multiple evidence strands pointing to those cytokines playing a primary role in the pathogenesis of NE [20, 21, 24, 25]. Nevertheless, our knowledge of the role of cytokines in the severity of NE disease remains limited. Therefore, in the current work we tested the hypothesis that patients with NE have a markedly different serum cytokine profile to healthy controls by screening both groups of subjects for serum concentrations of 48 cytokines associated with immune responses to infection and we link these responses to markers of pathology experienced by patients. Our findings support previous work in that a more extreme inflammatory cytokine profile was associated with markers of acute kidney injury and that this cytokine profile was more marked in younger patients.

93 **Materials and Methods**

94 *2.1. Subjects*

95 Serum samples were collected from 139 patients (117 males and 22 females) and controls 57 (21
96 males and 36 females). Clinical records (including clinical pathology records) were also collated
97 for these patients. Additionally, clinical laboratory test results such as serum levels of potassium
98 ion triglycerides, cholesterol, very low density cholesterol (VLDCL), low density cholesterol
99 (LDCL) and high density cholesterol (HDCL), routinely done upon hospitalization were
100 collected. Data were collected during the acute (VLDCL1, LDCL1 and HDCL1) and
101 convalescent (VLDCL2, LDCL2 and HDCL2) phases of HFRS. The diagnosis of HFRS was
102 established based on clinical presentation and was serologically confirmed by the detection of
103 anti-hantavirus antibodies. Samples were collected following the standard operating procedure
104 protocol in the hospital for the diagnosis of hantavirus infection and stored at -80^{0}C until used.

Commented [SK1]: Here I added. We did not conduct those test. We used these data as part of the clinical lab data ok

105 *2.2. Ethics Statement*

106 The ethics committee of the Kazan Federal University approved this study, and signed informed
107 consent was obtained from each patient and controls according to the guidelines adopted under
108 this protocol (protocol 4/09 of the meeting of the ethics committee of the KSMA dated
109 September 26, 2019).

110 *2.7. Hantavirus ELISA*

111 The Hantagnost diagnostic ELISA kit (Institute of Poliomyelitis and Viral Encephalitis,
112 Moscow, Russia) was used to determine hantavirus-specific antibody titers as per manufacturer's
113 instructions. Briefly, NE patient and control sera were diluted 1:100 (PBS) and incubated for 60
114 min at 37°C in a 96-well plate with pre-adsorbed hantavirus antigens. Following washes (3x;
115 0.5% Tween20 in PBS, PBS-T), wells were incubated with anti-human-IgG-HRP conjugated
116 antibodies (1:10000 in PBS-T, Amerixan Qualex Technologies, USA) for 30 min at 37°C. Post,
117 incubation and washes (3x; 0.5% Tween20 in PBS), wells were incubated with 3,3',5,5'
118 Tetramethylbenzidine (Chema Medica, Moscow, Russia). The reaction was stopped by adding an
119 equal amount of 10% phosphoric acid (TatKhimProduct, Kazan, Russia). Data were measured

using a microplate reader Tecan 200 (Tecan, Switzerland) at OD_{450} with reference OD_{650}. OD_{450} values higher than 0.5 were considered positive results.

2.11. Multiplex Analysis

Serum levels of 48 analytes were analyzed using Bio-Plex (Bio-Rad, Hercules, CA, USA) multiplex magnetic bead-based antibody detection kits following the manufacturer's instructions. Multiplex kits, Bio-Plex Pro Human Cytokine 21-plex, and Bio-Plex Human Cytokine 27-plex panels were used in the study. Serum aliquots (50 µl) were analyzed where a minimum of 50 beads per analyte was acquired. Median fluorescence intensities were collected using a Luminex 100 or 200 analyzer (Luminex, Austin, TX, USA). Each sample was analyzed in triplicate and the resulting data were analyzed with MasterPlex CT control software and MasterPlex QT analysis software (MiraiBio, San Bruno, CA, USA). Standard curves for each cytokine were generated using standards provided by the manufacturer. Data were analyzed using MasterPlex CT control software and MasterPlex QT analysis software (MiraiBio, Alameda, CA, USA).

2.17. Statistical Analysis

Clinical symptoms analysis ~~*using* χ^2 *criterion*~~. Analysis of clinical symptoms (presence or absence of each symptom in turn) was by loglinear model selection of contingency tables in IBM SPSS Statistics version 24, based on maximum likelihood. Initially, full factorial models comprising symptom (2 levels, presence/absence) x sex (2 levels, male/female) x age (two levels, ≤ 40/>40 years old) were fitted, and then simplified by the backward selection procedure to generate minimum sufficient models (MSM) for which the likelihood ratio of χ^2 was not significant, indicating that the model was sufficient in explaining the data. The importance of each individual term in MSMs was assessed by the probability that its exclusion would alter the model significantly, and relevant χ^2 values with associated probabilities ~~are~~ provided. Quantitative clinical data were analyzed by multivariate GLM models in R version 2.2.1 (R Core Development Team).

Analysis of individual cytokines. Preliminary analysis of individual cytokines was done using the non-parametric Mann–Whitney test with Benjamini-Hochberg (BH) adjustment for multiple comparisons using R language for statistical computing (R Core Development Team). The threshold used for statistical significance was $p < 0.05$.

149 Cytokine analysis using Principal Components Analysis (PCA). Since the data comprised
150 values for 48 different cytokines and their receptors, in order to avoid the risk of Type I and
151 Type II statistical errors, we first conducted a PCA in IBM SPSS vs 24. The major principal
152 components (PCs) responsible for the majority of variance in the data were then subjected to
153 statistical analysis via two Generalized Linear Models (GLMs) in R version 2.2.1.

154 PC1 and PC2 did not conform to Gaussian distributions and all attempts to fit models with
155 normal error structures failed to generate normally distributed residuals. The best-fit distributions
156 were negative binomial. Therefore, the data were transformed by the addition of 0.85 to PC1
157 values and 1.38 to PC2 values to convert all records to positive values, then multiplied by 100 to
158 avoid decimals, and rounded off to the nearest integer. These values were then used in GLMs.

159 Summary data are presented as arithmetic means of the PC and standard errors of the
160 mean (S.E.M.). We fitted models in R with PC1 or PC2 as the dependent variables. Each
161 subject's age was fitted as a covariate. Sex (at two levels, males and females), and subject's
162 status (at two levels, patient or control) were fitted as fixed explanatory factors. Full factorial
163 models that converged satisfactorily were simplified using the backward selection procedure and
164 tested for significance at each step using deletion of terms beginning with the highest order
165 interaction by comparing models with or without that interaction (3-way interaction). This was
166 followed by models based on main effects plus 2-way interactions, and deletion of 2-way
167 interactions in turn, and so on until each main effect was evaluated in a model that only
168 comprised all main effects. Models were evaluated by the likelihood ratio (*LR*) and associated
169 probability of rejecting the null hypothesis. Minimum sufficient models (MSMs) were then fitted
170 (all significant main effects and any significant interactions) and the process was repeated to
171 obtain values for changes in 2 x log-likelihood, test statistic (likelihood ratio [LR]) and
172 probabilities.

173 The acceptability of GLMs was evaluated through the goodness of fit of residuals from
174 MSMs through Q-Q plots and through estimation of the total variance accounted for by the
175 model. The percentage of variance accounted for by each significant main effect or interaction
176 was calculated as recommended by Xu (2003), and reported earlier by Behnke *et al.,* (2008) and
177 more recently by Grzybek *et al.,* (2015a).

178 Finally, we fitted a multivariate model in R in which we included PC1, PC2, age and sex
179 as explanatory factors and six markers of pathology that were available for both patients and

Formatted: Underline

Formatted: Underline

180 controls, as the dependent variables. In order to illustrate how markers of pathology vary in
181 relation to increasing values of PC1 and PC2, we divided the values of each into four ranges and
182 that of the controls, as follows:
183 PC1
184 Control subjects range = -0.827 to -0.367
185 Patients range 1 = -0.703 to -0.369 (all within the control range, n= 57)
186 Patients range 2 = -0.344 to +0.973 (marginally above control range, n=52)
187 Patients range 3 = +1.022 to +1.780 (much higher than control range, n=17)
188 Patients range 4 = +2.035 to 4.195 (very much higher than control range, n=11)
189
190 PC2
191 Control subjects range = -0.723 to -0.070 (with one extreme exception at 0.548)
192 Patients range 1 = -1.352 to -0.086 (all within the control range, n= 58
193 Patients range 2 = -0.074 to +0.492 (marginally above control range, n=46)
194 Patients range 3 = +0.506 to +1.689 (much higher than control range, n=27)
195 Patients range 4 = +1.845 to 7.401 (very much higher than control range, n=6)
196

197 **Results**

198 *Clinical presentation of NE cases*.

199 HFRS diagnosis was based on clinical presentation and epidemiological data as well as
200 serological confirmation. The average hospitalization period was 9.4±0.4 days and the average
201 duration of the febrile period 6.8±0.1 days. Clinical and demographic data are summarized in
202 Table 1.

203 Table 1. Demographic, clinical and laboratory information for NE.

Variables	Value
Age (years)	38 ±12.9
Sex (M/F)	117/22
Age M (years)	38.4±12
Age F (years)	47.4±14
Mild form HFRS (%)	10.07
Moderate form HFRS (%)	58.23
Severe form HFRS (%)	23.72
Mild HFRS M/F	17/8 (14.5%/36.4%)
Moderate HFRS M/F	71/11 (60.7%/50%)
Severe HFRS M/F	29/3 (24.8%/13.6%)
Antibody titer (1st)	1:200
Antibody titer (2nd)	1:800
Hospitalization (days)	9.4±4.7

204

205 The clinical form of the disease was classified as mild, moderate or severe. There were more
206 male patients as compared to female diagnosed with NE. The mild form was characterized by
207 fever (38^{0}C), oliguria (900 ml/day; 39% of patients), micoproteinuria (0.1 g/L), a normal level of
208 urea (1.7-8.3 mM/L), and increased levels of creatinine (up to 130 mkM/L). Hemorrhagic
209 syndrome presented as nose bleeding in 5% of patients. Patients with the moderate form of
210 HFRS had fever (39.5^{0}C), headache, frequent vomiting and abdominal pain, back pain, multiple
211 petechias, oliguria (300 ml/day; 68.6%), and levels of urea and creatinine up to 18 mM/L and
212 300 mkM/L, respectively. The moderate form of HFRS was characterized by pronounced
213 hemorrhagic syndrome (10.2%), which included nose bleeding (8.8%) and petechias (5.8%). In
214 contrast, patients with the severe form of HFRS had complications such as shock, acute
215 cardiovascular insufficiency (22.5%), hemorrhages (74.1%), oliguria (less than 300 ml/day;
216 100%) or anuria (54.8%), and levels of urea and creatinine higher than 18.5 mM/L and 300
217 mM/L, respectively. In addition, 16.1% of patients required hemodialysis. Hemorrhagic

218 syndrome in these patients included nose bleeding (67.7%), hemorrhages (38.7%), as well as
219 scleral hemorrhages (25.8%).
220 Next, we sought to determine whether frequency of clinical symptoms differed depending on
221 sex and age of NE (Table 2). As expected, the severity of symptoms worsened with the disease
222 class (class 1- mild; class 2- moderate and class 3 – severe). We also found a higher frequency of
223 hemorrhagic (nose bleeding and petechia) and gastro-intestinal (diarrhea and abdominal)
224 symptoms in male as compared to female patients. Additionally, symptoms of renal dysfunction
225 (anuria and oliguria) as well as fog in eye were more often described in male as compared to
226 female patients. Only one symptom, cough, was found more frequently in females as compared
227 to male subjects.
228

229 Table 2. Prevalence of clinical symptoms according to severity of disease and age.

230

Symptom	Symptom Class	Symptom Severity Prevalence [CL_{95}][¶¶]	Sex	Sex Prevalence [CL_{95}]	Age class	Age Prevalence [CL_{95}]
Nose bleed	1	**4.0** [0.21-19.56]	Male	**16.2**[11.35-22.47]	1	**17.2** [9.27-29.07]
	2	**9.9** [4.44-19.94]	Female	**0.0** [0.0-15.17]	2	**7.7** [3.59-15.03]
	3	**30.3** [18.62-44.92]				
		*		*		**NS**
Petechia	1	**0.0** [0.0-13.36]	Male	**18.8** [13.51-25.42]	1	**23.0** [13.63-35.72]
	2	**4.9** [1.47-13.63]	Female	**4.5** [0.24-22.21]	2	**5.8** [2.46-12.31]
	3	**57.6** [42.87-71.27]				
		***		**NS**		**
Scleral bleed	1	**0.0** [0.0-13.36]	Male	**6.8** [3.89-11.63]	1	**6.9** [2.45-16.61]
	2	**2.5** [0.38-10.00]	Female	**0.0** [0.0-15.17]	2	**3.8** [1.27-9.77]
	3	**18.2** [9.31-31.91]				
		**		**NS**		**NS**
Bleeding	1	**4.0** [0.21-19.56]	Male	**29.1** [22.67-36.33]	1	**32.2** [21.14-45.12]
	2	**17.3** [9.56-28.64]	Female	**4.5** [0.24-22.21]	2	**13.5** [7.73-21.77]
	3	**60.0** [45.93-74.10]				
		***		**		**NS**
Cough	1	**28.0** [13.37-47.97]	Male	**5.1** [2.66-9.50]	1	**3.4** [0.69-11.86]
	2	**7.4** [2.85-16.76]	Female	**31.8** [15.18-54.65]	2	**19.2** [12.41-28.34]
	3	**0.0** [0.0-8.04]				
		***		**		*
Diarrhoea	1	**12.0** [3.36-30.31]	Male	**35.0** [28.14-42.53]	1	**35.6** [24.26-48.58]
	2	**32.1** [21.46-44.55]	Female	**31.8** [15.18-54.65]	2	**23.1** [15.34-32.69]
	3	**42.2** [28.73-57.13]				
		NS		**		**NS**
Vomiting	1	**8.0** [1.45-25.59]	Male	**35.0** [28.14-42.53]	1	**41.4** [29.15-54.36]
	2	**34.6** [23.60-47.03]	Female	**31.8** [15.18-54.65]	2	**23.1** [15.34-32.69]
	3	**54.5** [39.81-68.37]				
		**		**NS**		*
Nausea	1	**36.0** [19.57-56.08]	Male	**57.3** [49.76-64.51]	1	**41.4** [29.15-54.36]
	2	**48.1** [35.63-60.69]	Female	**31.8** [15.18-54.65]	2	**23.1** [15.34-32.69]
	3	**78.8** [64.27-88.59]				
		**		**NS**		*
Abdominal pain	1	**28.0** [13.37-47.97]	Male	**67.5** [60.08-74.20]	1	**70.1** [57.20-80.62]
	2	**59.3** [46.76-71.06]	Female	**27.3** [12.61-50.00]	2	**46.2** [36.45-56.34]

	3	**90.0** [78.91-96.71]				
		***		**		*
Back pain	1	**44.0** [25.60-64-25]	Male	**65.8** [58.32-72.55]	1	**69.0** [56.05-79.75]
	2	**63.0** [50.48-74.10]	Female	**59.1** [38.26-77.78]	2	**57.7** [47.53-67.39]
	3	**84.8** [71.35-93.03]				
		**		**NS**		**NS**
Anuria	1	**0.0** [0.0-13.36]	Male	**14.5** [9.91-20.59]	1	**17.2** [9.27-29.07]
	2	**0.0** [0.0-6.07]	Female	**4.5** [0.24-22.21]	2	**5.8** [2.46-12.31]
	3	**54.5** [39.81-68.37]				
		***		***		***
Oliguria	1	**20.0** [8.23-39.84]	Male	**72.6** [65.46-78.85]	1	**75.9** [63.12-85.47]
	2	**70.4** [57.93-80.52]	Female	**45.5** [26.05-66.17]	2	**55.8** [45.60-65.46]
	3	**100.0**[91.96-100.0]				
		*		**NS**		*
Fog eye¶	1	**16.0** [5.66-35.74]	Male	**54.7** [47.19-62.00]	1	**59.8** [46.79-72.00]
	2	**44.4** [32.65-56.96]	Female	**13.6** [3.83-33.82]	2	**28.8** [20.64-38.90]
	3	**81.8** [68.09-90.69]				
		***		***		***

***** *P* = 0.05-0.01; ****** *P*=0.099-0.001; ******* *P*<0.001

For severity classes 1- mild, 2- moderate and 3- severe. The sample sizes for each class were 25, 81 and 33 respectively. Number of male patients =117 and females=22. Number of patients for age classes 1 (≤40 years old) and 2 (>40 years old) were 87 and 52. respectively.

Prevalence is the percentage (%) of subjects showing the symptom in the relevant data subset. CI_{95} are the 95% confidence limits. For further details see text.

In the case of fog eye there were also two significant interactions. Age x sex P=0.017 and sex x severity. P<0.001.

Commented [RT2]: are these the initial chi squared (univariate) analysis? Not clear from the methods what/where the P values in this table have been calculated...

243 We acknowledge, that the number of samples in sex groups differ, having more male as
244 compared to females, which is characteristic for NE [1, 26]. Therefore, this discrepancy in
245 number of samples could be a factor affecting the analysis.
246 When NE symptoms were analyzed based on age of the patient, we found that younger
247 patients (≤40 years old) had a higher frequency of hemorrhagic (petechia), gastro-intestinal
248 (vomiting, nausea, abdominal pain) and eye fog symptoms as compared to older (>40 years old)
249 NE. Also, younger patients presented with kidney dysfunction (anuria and oliguria) symptoms
250 more often as compared to older NE. Cough was the only symptom which was more frequent in
251 older as compared to younger NE patients. These data indicate that clinical presentation of NE
252 depends on sex and age of the patient. Although multiple factors could contribute to variation of
253 NE, activation of cytokines could play a substantial role.
254

255 *Analysis of cytokine levels*
256 The mean values of cytokine and receptor levels detected in the sera are given in Table 3,
257 which also shows the arithmetic difference between values in patients and the control group, as
258 well as the relative change in value between these groups (mean value of patients divided by that
259 of controls). With the exception of IL-1α and CCL27, the mean levels of all the other cytokines
260 were arithmetically higher in patients relative to controls.
261 ~~Table 3~~
262 Table 3. Mean values (± S.E.M.) for all cytokines and receptors and the arithmetic difference
263 between the mean values of patients and control subjects.
264 In order of the magnitude of the change

	Patients	Controls	Mean difference	X change	Mann-Whitney U test
	(n=139)	(n=57)	Patients minus controls	Patients/controls	P value
IL-1α	0 62±0.08	1.292±0.12	-0.67	0.48	0.0001*
CCL27	69.89±7.03	125.19±10.00	-55.303	0.56	0.0001*
CXCL12	45.85±9.41	36.284±6.14	9.563	1.26	0.38318
CXCL1	65.07±6.034	51.481±4.88	13.585	1.26	0.66127
CCL7	29.12±2.75	18.724±3.04	10. 394	1.56	0.02323*
IL-8	63.04±12.26	37.918±15.57	25.123	1.66	0.00011*
IL-16	215.12±34.78	122.418±9.91	92.703	1.76	0.04059*
TNFβ	2.23±1.16	1.196±0.27	1.035	1.87	0.23723

SCF	72.24±7.04	33.774±2.39	38.464	2.14	0.00066*
IFN-α2	21.574±3.40	8.958±0.90*	12.616	2.41	0.00012*
TRAIL	43.553±4.61	16.397±2.52	27.156	2.66	0.00002*
IL-3	201.265±26.20	66.724±5.96	134.541	3.02	0.00015*
IFN-γ	100.881±15.60	32.643±4.32	68.238	3.09	0.0001*
IL-18	27.441±3.10	8.631±1.48	18.78	3.18	0.0001*
IL- 12p40	288.664±32.77	88.116±12.51	200.548	3.28	0.0001*
MIF	518.034±65.86	145.137±25.43	372.897	3.57	0.0001*
LIF	8.739±2.68	2.404±0.44*	6.335	3.64	0.00004*
M-CSF	5.809±2.02	1.491±0.14	4.318	3.90	0.0001*
G-CSF	32.999±2.22	8.074±0.92	24.925	4.09	0.0001*
HGF	402.173±37.50	97.191±13.56	304.982	4.14	0.0001*
IL-1 ra	141.589±30.78	31.442±5.42	110.147	4.50	0.0001*
IL-2RA	133.927±15.07	28.862±3.24	105.065	4.64	0.0001*
SCGF - b	8486.585±868.14	1564.75±242.42	6921.838	5.42	0.0001*
CCL11	89.706±10.47	15.50±2.90	74.21	5.79	0.0001*
CCL2	89.357±25.09	13.02±1.33	76.34	6.85	0.0001*
IL-7	14.519±3.14	2.08±0.40	12.44	6.98	0.0001*
IL-5	8.067±1.19	1.02±0.24	7.043	7.88	0.0001*
GM-CSF	23.25±4.03	2.58±0.69	20.67	9.01	0.0001*
IL-15	53.60±13.06	5.40±0.86	48.196	9.92	0.0001*
IL-12(p70)	38.73±5.5	3.73±0.48	35	10.38	0.0001*
TNF-α	43.66±9.10	4.17±0.76	39.495	14.48	0.0001*
VEGF	175.55±25.20	15.15±2.45	160.402	11.59	0.0001*
B-NGF	8.54±3.96	0.73±0.06	7.809	11.67	0.0001*
IL-6	39.42±5.95	2.90±0.65	36.516	13.58	0.0001*
CXCL9	1797.09±253.08	124.22±18.93	1672.868	14.47	0.0001*
FGF b	19.53±2.12	1.29±0.31	18.233	15.10	0.0001*
IL-2	29.14±9.54	1.78±0.30	27.357	16.33	0.0001*
IL-10	58.92±11.58	3.56±0.65	55.361	16.55	0.0001*
IL-4	19.01±2.54	1.10±0.09	17.906	17 16	0.0001*
IL-17	42.65±8.87	2.27±0.56	40.376	18.76	0.0001*
IL-1β	15.81±2.07	0.81±0.15	15.007	19.60	0.0001*
IL-9	96.2±22.78	3.50±0.51	92.698	27.47	0.0001*
IL-13	36.93±5.71	1 34±0.13	35.588	27.60	0.0001*
CCL3	47.83±8.62	0.97±0.34	46.857	49.31	0.0001*
CCL5	3062.01±398.29	60.99±8.72	3001.024	50.21	0.0001*
PDGF-bb	8105.64±5756.86	144.56±23.81	7961.073	56.07	0.0001*
CXCL10	3497.04±390.01	49.05±7.17	3447.989	71.29	0.0001*
CCL4	1020.56±144.66	10.27±2.13	1010.289	99.37	0.0001*

265

266 n=numbers of control subjects in this case is 56.
267 Mean difference - the arithmetic difference between the mean level of each cytokine in
268 patients and controls (patient value minus control value). Numbers in red are negative values
269 indicating that the level of the cytokine was higher in controls relative to patients. Those in
270 black show cytokine levels higher in patients compared to controls
271 X change - the ratio of the mean value in patients and that in controls (patient value divided by
272 the control value). Here numbers in red have values less than 1, indicating that the level of the
273 cytokine in each case was lower in patients than in controls. Numbers in blue show cytokine
274 levels >1 to 5 times higher in patients relative to controls. Numbers in black show cytokine
275 levels >5 to 10 times higher in patients relative to controls and those in green show cytokine
276 levels >10 times higher in patients relative to controls.
277 *- significantly different cytokines between NE and controls, $p < 0.05$, Mann-Whitney U test
278 $p<0.05$
279 Analysis was based on PCA to avoid statistical errors arising from multiple tests, as explained
280 above (Materials and Methods). PCA identified in total 13 components as quantifiable
281 (collectively accounting for 80% of variance). PC1 was the dominant component accounting for
282 almost a quarter of total variance (23.1%), and PC2 explained the next 15.3%. Between them,
283 therefore these two accounted for 38% of the variance. None of the other PCs accounted for
284 more than 7% of variance, and these were not studied further.
285 Twenty eight of the cytokines and receptors contributed positively to PC1 (Fig. 1), with
286 values ranging from 0.898 to 0.101. The greatest positive contribution was from IL-1β (0.898),
287 IL-4 (0.862), IL-12 (0.828), CCL5 (0.809) and GM-CSF (0.801). Three cytokines (CXCL1, IL-
288 1α and CCL27) made negative contributions to PC1 (-0.109, -0.307 and -0.417, respectively).
289 Twenty-seven cytokines and receptors contributed positively to PC2, the greatest contributions
290 being from IL-3 (0.873), SCF (0.805), CCL7 (0.794), TRAIL (0.793), IFNγ (0.771), IL-1ra
291 (0.763) and IL-12p40 (0.718). There were nine negative contributions greater than -0.1, as shown
292 in Fig. 1.
293
294 *Frequency distributions of PC1 and PC2*
295 The frequency distributions of PC1 and PC2 are illustrated in Figs 2A and 2B, respectively.
296 The values of PC1 in controls did not exceed -0.3, and 56 patients also had values in the control
297 range (Fig. 2A). The remaining patients had higher values, the first of which form an extension
298 to the peak that includes controls, and then perhaps up to 2-3 peaks at higher values of PC1.
299 These suggest different degrees of responsiveness to infection. The difference between patients
300 and controls was highly significant (GLM with negative binomial errors, main effect of subject

301 status, $LR_{1,189}$=108.75, P<0.0001), accounting for 5.22% of the variance in the data. Fig. 2B
302 shows that values of PC2 in controls, with just one exception, were restricted to values less than -
303 0.06. Twenty-five patients had values in the control range and some even lower and, as with
304 PC1, there appeared to be several clusters in patients at higher values. The difference between
305 patients and controls was highly significant (GLM with negative binomial errors, main effect of
306 subject status, $LR_{1,190}$=26.378, P<0.0001), accounting for 1.2% of the variance in the data.
307

308 *Relationship of PC1 with PC2*

309 The relationship of PC1 to PC2 is shown in Fig. 3, where it can be seen that values for control
310 subjects cluster tightly in the bottom left-hand corner. This figure shows that many of the
311 subjects with high PC1 values kept PC2 values in the control range, although some with
312 relatively low PC1 values had high PC2 values, outside the control range. Moreover, there were
313 just two patients with very high values for both. In order to provide more clarity of the
314 clustering, part of this figure, spanning the range from -1.0 to +1 for PC1, and -1.5 to 2 for PC2,
315 is magnified in Fig. 6B. If we take the control values as -0.827 to -0.367 for PC1 and -0.723 to-
316 0.076 for PC2, only 15 (10.8%) patients had PC1 and PC2 values that lie in this area on the
317 figure, and therefore 89.2% had increased serum levels of both the cytokines reflected in PC1
318 and PC2.
319

320 *Age-dependent variation in PC1 and PC2*

321 The mean value of PC1 in male (-0.642 ± 0.016) and female (-0.640 ± 0.017) controls was
322 almost identical. Among patients, the mean value of PC1 was arithmetically higher in male
323 subjects (0.306 ± 0.104) compared with females (-0.011 ± 0.177). However, the S.E.M.s are
324 large and therefore, with age taken into account, there was no overall significant difference
325 between the sexes (GLM with negbin errors, main effect of sex, $LR_{1,188}$=0.579, P=0.447) and no
326 significant interaction between subject status (patient or control) and sex ($LR_{1,185}$=0.399,
327 P=0.528). *Post hoc* analysis by the Mann-Whitney U test confined to patients confirmed that
328 PC1 did not differ between the sexes ($U_{116,21}$ =975.0, P=0.147). Nevertheless, many of the high
329 values for PC1 were from male subjects. In 95% of female subjects for which PC1 could be
330 calculated, PC1 ranged from -0.656 to 0.947, and with only one exception of a female subject

with a value of 2.547. In contrast, among male subjects 28 subjects (24.1%) had values exceeding 0.947, and seven (6.0%) values exceeding 2.547.

The data in Fig. 2C show that there is a tendency for younger patients to have high values of PC1, and with subject status taken into account, there was a significant effect of host age (GLM with negbin errors, subject status x age, $LR_{1,189}$=7.524, P=0.0061) accounting for 0.379% of the variance in the data. As patients aged, their PC1 values decreased (β=-0.02, R^2=0.058, t=-2.873, P=0.005). However, among controls, there was a very subtle increase in PC1 values with age but this was not significant (β=0.001, R^2=0.025, t=1.167, P=0.248). These different slopes in the relationship between age and PC1 values generated a significant 2-way interaction (GLM with negbin errors, subject status x age, $LR_{1,188}$=6.136, P=0.0132) accounting for 0.311% of the variance in the data.

For PC2, the values in control subjects were also very similar in the two sexes (males = -0.422 ± 0.037, females = -0.410 ± 0.041). Although this time the values were arithmetically higher for female patients (0.278 ± 0.211) compared with males (0.146 ± 0.108), the difference between the sexes was not significant (GLM with negbin errors, main effect of sex, $LR_{1,188}$=0.129, P=0.719), nor was the 2-way interaction significant (subject status x sex, $LR_{1,185}$ <0.001, P=0.993). *Post hoc* analysis by the Mann-Whitney *U* test confined to patients confirmed that PC2 did not differ between the sexes ($U_{116,21}$ = 1397.0, P=0.285).

The age-distribution of PC2 is illustrated in Fig. 2D. Neither the main effect of age ($LR_{1,188}$=0.992, P=0.319) nor the 2-way interaction, age x subject status ($LR_{1,185}$=1.500, P=0.221) were significant in the case of PC2. The slope for patients is β= 0.009 (R^2=0.010, t=1.160, P=0.248) and that for the controls β= -0.003 (R^2=0.033, t= -1.338, P=0.187). Two huge outliers can also be seen in Fig. 2D, presumably subjects that have over-reacted.

Age-dependent variation in specific cytokines

To examine how individual cytokine levels differ between age classes, we separated patients into two groups: younger (≤40 years old) and older (>40 years old) (Figure 4; Table S1). The relative response of each age class to their respective controls was calculated from the ratio of these responses (i.e mean values in age class 1[patients minus controls] divided by mean value in age class 2, [patients minus controls], and these are illustrated in the form of a heat map in Fig. 4). The majority of cytokines were upregulated in both groups of patients as compared to

362 controls (positive values in Table S1; column: Arithmetic difference), suggesting that
363 pathogenesis of the disease was mainly similar in both groups. Post Hoc~~The~~ Mann-Whitney
364 analysis revealed that 43 cytokines differed significantly between NE and controls in the younger
365 age class, while among older subjects 41 differed.

366 Among the resulting ratios twenty six cytokines were higher, while twenty two cytokines
367 were lower in younger as compared to older NE (Table S1; column: X difference). One cytokine
368 in particular, IL-8, had a particularly high value indicating that young male subjects responded
369 much more intensively compared to their age matched controls, than did older subjects (in older
370 subjects the mean levels of IL-8 were only marginally higher than those of their age matched
371 controls). However, there were three cytokines (CXCL1, CXCL12 and TNFβ), which were lower
372 in the sera of younger patients as compared to their age-matched controls, while in older patients
373 the levels of these cytokines were higher than among their respective controls. Of note, only two
374 cytokines, IL-1α and CCL27, were lower in both age classes relative in each case to their age-
375 matched controls. Post Hoc a~~A~~nalysis using Mann-Whitney U test identified three cytokines
376 which were significantly higher in younger as compared to older NE (Table S1).

377

378 *The relationship of PC1 and PC2 to measures of pathology.*

379 We fitted a multivariate model in R, with six measures of pathology as the dependent
380 variables. In the first run of this model sex was not a significant factor (Pillai trace statistic =
381 0.043, $F_{6,167}$=1.24, P=0.287). Therefore, sex was removed from the model and all remaining
382 explanatory factors retained significance. The strongest effect was from PC1 (Pillai trace statistic
383 = 0.233, $F_{6,169}$=8.53, P<0.0001). Age (Pillai trace statistic = 0.089, $F_{6,167}$=2.74, P=0.014) and
384 PC2 (Pillai trace statistic = 0.076, $F_{6,167}$=2.31, P=0.036) had weaker effects on the six dependent
385 variables (the six measures of pathology).

386 In order to illustrate these effects of PC1 and PC2 on measures of pathology, each PC was
387 divided into four ranges and plotted alongside the values from control subjects (Fig. 7). Thus,
388 with age and subject status (patient and control) taken into consideration, for potassium levels,
389 the effect of PC1 was positive and significant (β= 3.284, t=6.605, P<0.0001), while that of PC2
390 was negative and significant (β= -1.259, t= -2.738, P=0.0068). The levels of triglycerides did not
391 vary significantly with PC1 or PC2 despite the higher means when age and subject status had
392 been controlled for. Cholesterol levels did not vary significantly with PC1 but showed significant

393 negative decline with increasing values of PC2 (β= -0.375, t= -2.728, P=0.0070). Neither PC1
394 nor PC2 affected the levels of VLDCL1 significantly. The levels of LDCL1 varied positively
395 with increasing PC1 (β= 0.402, t= 2.710, P=0.0074) and negatively with increasing values of
396 PC2 (β= -0.334, t= -2.43, P=0.0160), while those of HDCL1 fell significantly with increasing
397 values of PC1 (β= -0.252, t= -4.402, P=0.0001) but did not vary significantly with PC2.
398

399 **Discussion**

400 Cytokines play an important role in the pathogenesis of NE [20, 21]. We have previously
401 demonstrated upregulation of pro-inflammatory cytokines in NE patients, including increased
402 levels of CXCL8 and IL-10 as compared to controls [21]. Previously, we have shown also that
403 serum TNFα and IL-1β were upregulated in severe HFRS [20] and we have demonstrated that
404 levels of IL-6, CXCL10, CCL2 and CCL3 are associated with clinical presentation of the
405 disease. In this earlier study, the serum level of only a limited number of cytokines was analyzed.
406 Therefore, building on our previous work, in the current analysis we included 48 cytokines and
407 receptors, including leukocytes, chemokines, growth factors as well as interferons and
408 proinflammatory cytokines. We found marked changes in the levels of a large number of
409 cytokines especially in subjects with the severe form of NE as compared to mild and moderate
410 forms of the disease at the febrile stage of the disease.

411 The results here demonstrate that the cytokine profile does indeed vary with disease with a
412 pro-inflammatory profile (PC1) being associated with several markers of acute kidney injury
413 (hyperkalaemia, oliguria, elevated creatinine and perturbations in cholesterol ratio). This pro-
414 inflammatory profile was more marked in younger patients, a finding that is concordant with the
415 known over-representation of younger patients in those with clinical disease, and the known
416 higher prevalence of hantavirus infection in younger compared with older patients. [31-33]. It
417 has been suggested that "cytokine storm" best explains the pathogenesis of hantavirus infection
418 [22, 25]; however, little is known about how serum cytokine levels vary with host age. NE is
419 diagnosed in patients of all ages [16, 34], however, it appears that recovery is more prolonged in
420 young female patients [35], and young male patients have a higher risk of developing serious
421 complications of the central nervous system [31]. The mechanisms underlying these serious
422 consequences remain largely unknown but our findings of an association between pro-
423 inflammatory cytokines and the young age of patients could provide an explanation. This

424 activation of the pro-inflammatory profile fits the “cytokine storm” model, where strong
425 activation of cytokines is linked to tissue damage and, potentially a fatal outcome [36]. Multiple
426 cytokines and chemokines, such as IL-1β, IL-6, CXCL10, CCL2, CCL11, G-CSF and GM-CSF,
427 have been shown to be associated with cytokine storms [37]. These cytokines we found
428 upregulated in young patients (Supplemental Table 1), suggesting their contribution to the
429 pathogenesis of the disease in this NE subset of the study group.

430 A high male to female ratio in the disease has been demonstrated in multiple studies [16, 33,
431 38]. Krautkramer et al suggested that a higher risk of exposure among male compared to female
432 subjects may explain the male bias in NE diagnoses [39]. In another study, the difference
433 between male and female subjects in the risk of contracting hantavirus infection was
434 hypothesized to be attributable to sex-related differences in expression of various estrogen
435 receptors [40]. The role of cytokines in sex-associated pathogenesis of hantavirus infection has
436 been demonstrated by Klingstrom et al where high levels of IL-8 and CXCL10 were identified
437 in male as compared to female NE [41] . Our results concur with the results of this study in that
438 we also found that the levels of IL-8 and CXCL10 in NE differ between the sexes. One of the
439 most intriguing findings in our study was a substantial increase in IL-8 level in the serum of
440 younger as compared to older NE patients. This cytokine is a potent chemokine, attracting
441 neutrophils to the site of infection [42] and favors the formation of neutrophil extracellular traps
442 [43]. IL-8 exposed neutrophils have higher adhesion to endothelial cells [44], transendothelial
443 migration [45] and tissue damage [46]. IL-8 may cause tissue damage by releasing matrix
444 metalloproteases degrading extracellular matrix components [47]. Supporting the pathogenic role
445 of IL-8 in NE is data presented by Strandin et al, where a positive correlation between the serum
446 level of this cytokine and kidney dysfunction was demonstrated [48]. Increased serum levels of
447 IL-8 in NE were shown also by Sadeghi et al [49]. These authors demonstrated that cytokine
448 serum levels were positively correlated with creatinine and C reactive protein, indicators of
449 kidney dysfunction and inflammation. Our data expand understanding of the role of IL-8 in NE
450 pathogenesis by identifying that younger patients respond most intensively with this cytokine.
451 Therefore, we suggest that IL-8 may contribute to variation in clinical presentation in these
452 groups of patients.

453 In agreement with Klingstrom et al [41] , we found also that younger males had higher levels
454 of CXCL10 as compared to the same age group females (4330 vs 179, respectively). Male

455 subjects of both age classes had higher values than their respective age-matched controls (138.2
456 times higher than age-matched controls for younger males and 35.51 for the older males), while
457 the younger females did not respond as well with his cytokine (only 3.5 times higher than age
458 matched controls). In contrast, the older females responded almost as well as the males (72.5
459 times higher than age matched controls). It should be noted that the sex groups were unequal,
460 with more female as compared to male NE included. This is characteristic for NE as it is
461 diagnosed more often in male as compared to female subjects [1, 26]. Therefore, this discrepancy
462 in the number of samples could be a factor affecting the analysis. More samples from female NE
463 in future studies will strengthen the robustness of analyses and resulting conclusions as to the
464 role of sex in disease pathogenesis.
465
466 Although the levels of many of the cytokines that we measured were arithmetically higher in
467 male as compared to female NE, our study did not reveal overall a significant difference in PC1
468 and PC2 between the sexes. The overriding importance of age in the cytokine profiles likely
469 masks the complex interactions of host sex and age. A greater tendency towards a PC1 profile
470 was demonstrated in male patients in this study with a more detailed scrutiny of individual
471 cytokines indicating that the responses of young men and women differed in many cases to older
472 patients of the same sex. While this study was of a reasonable size it is likely that much larger
473 age and sex matched cohort studies will be necessary to fully characterize these differences.
474 Future studies would also need to take into account likely confounding factors such as the pre-
475 and post-menopausal status of female patients in their cytokine responses.
476 Aging has profound effects on the functioning of the immune system. Declining antibody
477 production is well documented in elderly populations [50], supporting the overall impaired
478 response typical of this sub-set of the population. Some of the more striking differences are
479 associated with reductions in T cell function and lowered IL-2 production [51, 52]. Lower IL-2
480 production in older as compared to younger NE patients was evident in our study (Figure 3;
481 Supplemental Table 3). Also, five common γ chain cytokine family members (IL-2, IL-4, IL-7,
482 IL-9 and IL-15) were found upregulated in younger patients (Figure 4; Table S2). As these
483 cytokines play a pivotal role in the development, survival, proliferation and differentiation of the
484 innate and adaptive immune responses [53], the lower level of these cytokines in older NE
485 patients could contribute to disease pathogenesis in this cohort of patients.

486 It should be pointed out that genetic factors could contribute to age dependent differences in
487 NE severity. Genetic mechanisms have been suggested also to play role in cytokine storms, the
488 leading factor in pathogenesis of hantavirus infection [25, 54, 55]. Recent studies of genetic
489 factors have implicated several *IL6* gene variations in pathogenesis of coronavirus infection 2019
490 (COVID-19) [56], a disease where severity has a strong association with the likelihood of a
491 cytokine storm [57]. Severity of influenza, another disease with cytokine storm based
492 pathogenesis, has been associated also with *IL1B* gene polymorphism [58]. The contribution of
493 genetic factors to pathogenesis of hantavirus infection has been investigated also [59]. Multiple
494 Human Leukocyte Antigen alleles (HLA) have been shown as connected to the severe form of
495 infection [60, 61]. Additionally, a haplotype associated with high production of TNF-α has been
496 correlated with the severe form of NE [62]. Also, *IL-1RA* allele 2 and *IL-1b* allele 2 have been
497 found to be less frequent in hantavirus infected patients as compared to seronegative controls
498 [63]. The contribution of these genetic factors to pathogenesis of NE could be modified by age,
499 environment and ethnicity [64-66].
500

501 We found some associations between biochemical laboratory data and cytokine PCs, notably,
502 the serum potassium levels (a marker of acute kidney injury) positively correlated with pro-
503 inflammatory PC1 cytokines. Interestingly, IL-1β, a major pro-inflammatory cytokine, has been
504 shown to inhibit the inwardly rectifying K+ channel in human proximal tubule cells [67, 68].
505 This could avert the intake of potassium leading to accumulation of this ion in the interstitial
506 space and in the serum. In the kidneys, IL-1β causes suppression of K+ channels which could
507 lead to lower reabsorption of Na+ [69] and glucose [69] , contributing to oliguria, the main
508 symptom of NE [14]. Interestingly, glucosurea is detected in PUUV infected patients and it has
509 been shown to correlate with disease severity [70]. Therefore, it could be suggested that the
510 markers of NE severity could be the result of the effects of pro-inflammatory cytokines on
511 kidney cell potassium transport function.

512 We found also that LDCL1 and HDCL1 have positive and negative associations with PC1
513 cytokines, respectively. Changes in the serum level of lipids have been demonstrated in
514 hantavirus infected patients [2, 71, 72]. Our results provide more data contributing to the
515 understanding of the role of lipids in pathogenesis of NE. The association between LDL and pro-
516 inflammatory cytokines has been demonstrated in multiple studies, where IL-1 and TNFα, the

Commented [JB3]: Should this be LDCL1 or just LDL, i.e generically expressed rather than specifically as in LDCL1?
SK: I agree, it should be LDL; however, technically cholesterol is lipid as well

517 main contributors to "cytokine storms" in model organisms or pathology were shown to increase
518 plasma low density lipids [73, 74]. In turn, low density lipids can activate production of IL-1β
519 and IL-18 by engaging Toll like receptors (TLRs) and triggering the formation of
520 inflammasomes [75]. In contrast, HDL were shown to have anti-inflammatory effects by
521 reducing expression of TLRs and reduced IFN receptor signaling [76]. Our data also support the
522 notion that HDL could have an anti-inflammatory effect as a negative association was found in
523 NE between HDL and PC1 cytokines. These data suggest that serum LDL and HDL could
524 contribute to the pathogenesis of NE; however, the mechanisms remain to be determined.
525 Conclusion. NE is an acute zoonotic disease which is characterized by kidney insufficiency
526 and hemorrhages. Although diagnosed in both sexes, higher male to female ratios in NE are
527 often reported [39]. The pathogenesis of the disease remains largely unknown; however,
528 excessive cytokine activation, known as "cytokine storm," is suggested to play a role. Finally, we
529 identified that high serum levels of potassium and LDL were associated with PC1 cytokines,
530 while serum HDL had an opposite association with the pro-inflammatory cytokine profile. These
531 associations between the PC1 cytokine profile and HDL, as well as LDL, are recorded for the
532 first time. Our data suggest an important role for pro-inflammatory cytokines in the pathogenesis
533 of NE, especially, in young patients.
534

535 **Acknowledgments**

536 This research was supported by the Kazan Federal University Strategic Academic Leadership
537 program.

538 **References**

539 1. Settergren, B.J.S.j.o.i.d., *Clinical aspects of nephropathia epidemica (Puumala virus*
540 *infection) in Europe: a review.* 2000. **32**(2): p. 125-132.
541 2. Mustonen, J., et al., *Nephropathia epidemica in Finland: a retrospective study of 126*
542 *cases.* Scandinavian journal of infectious diseases, 1994. **26**(1): p. 7-13.
543 3. Latus, J., et al., *Clinical course and long-term outcome of hantavirus-associated*
544 *nephropathia epidemica, Germany.* Emerging infectious diseases, 2015. **21**(1): p. 76.
545 4. Settergren, B., et al., *Clinical characteristics of nephropathia epidemica in Sweden:*
546 *prospective study of 74 cases.* Rev Infect Dis, 1989. **11**(6): p. 921-7.
547 5. Rasche, F.M., et al., *Thrombocytopenia and acute renal failure in Puumala hantavirus*
548 *infections.* Emerging infectious diseases, 2004. **10**(8): p. 1420.
549 6. Radonić, R., et al., *Thrombotic thrombocytopenic purpura and hemorrhagic fever with*
550 *renal syndrome: possible dilemma in differential diagnosis.* Acta medica Croatica:
551 casopis Hravatske akademije medicinskih znanosti, 2003. **57**(5): p. 433-436.

552 7. Takala, A., et al., *Systemic inflammation in hemorrhagic fever with renal syndrome*
553 *correlates with hypotension and thrombocytopenia but not with renal injury.* Journal of
554 Infectious Diseases, 2000. **181**(6): p. 1964-1970.
555 8. Huang, C., et al., *Hemorrhagic fever with renal syndrome: relationship between*
556 *pathogenesis and cellular immunity.* Journal of Infectious Diseases, 1994. **169**(4): p. 868-
557 870.
558 9. Tang, Y.-M., et al., *Localization and changes of hemorrhagic fever with renal syndrome*
559 *virus in lymphocyte subpopulation.* Chinese medical journal, 1991. **104**(8): p. 673-678.
560 10. Saari, M., et al., *Nephropathia epidemica: the Scandinavian form of hemorrhagic fever*
561 *with renal syndrome.* Jama, 1977. **238**(8): p. 874-877.
562 11. Hentzien, M., et al., *Bioclinical Test to Predict Nephropathia Epidemica Severity at*
563 *Hospital Admission.* Emerging Infectious Diseases, 2018. **24**(6): p. 1045.
564 12. Settergren, B., *Clinical aspects of nephropathia epidemica (Puumala virus infection) in*
565 *Europe: a review.* Scandinavian journal of infectious diseases, 2000. **32**(2): p. 125-132.
566 13. Settergren, B., *Nephropathia epidemica (hemorrhagic fever with renal syndrome) in*
567 *Scandinavia.* Reviews of infectious diseases, 1991. **13**(4): p. 736-744.
568 14. Turčinov, D., et al., *Clinical and laboratory findings in patients with oliguric and non-*
569 *oliguric hantavirus haemorrhagic fever with renal syndrome: an analysis of 128 patients.*
570 Clinical microbiology and infection, 2013. **19**(7): p. 674-679.
571 15. Germash, E., et al., *The pathogenetic therapy of patients with a severe form of*
572 *hemorrhagic fever and acute kidney failure.* Terapevticheskii arkhiv, 1997. **69**(11): p. 26.
573 16. Khismatullina, N., et al., *Epidemiological dynamics of nephropathia epidemica in the*
574 *Republic of Tatarstan, Russia, during the period of 1997–2013.* Epidemiology &
575 Infection, 2016. **144**(3): p. 618-626.
576 17. Davidyuk, Y., et al., *Characterization of the Puumala orthohantavirus strains in the*
577 *northwestern region of the Republic of Tatarstan in relation to the clinical manifestations*
578 *in hemorrhagic fever with renal syndrome patients.* Frontiers in pharmacology, 2019. **10**:
579 p. 970.
580 18. Khaiboullina, S.F., et al., *Effects of tumor necrosis factor alpha on Sin Nombre virus*
581 *infection in vitro.* Journal of virology, 2000. **74**(24): p. 11966-11971.
582 19. Temonen, M., et al., *Cytokines, adhesion molecules, and cellular infiltration in*
583 *nephropathia epidemica kidneys: an immunohistochemical study.* Clinical immunology
584 and immunopathology, 1996. **78**(1): p. 47-55.
585 20. Baigildina, A.A., et al., *Inflammatory cytokines kinetics define the severity and phase of*
586 *nephropathia epidemica.* Biomarkers in medicine, 2015. **9**(2): p. 99-107.
587 21. Khaiboullina, S., et al., *Upregulation of IFN-γ and IL-12 is associated with a milder form*
588 *of hantavirus hemorrhagic fever with renal syndrome.* European journal of clinical
589 microbiology & infectious diseases, 2014. **33**(12): p. 2149-2156.
590 22. Easterbrook, J.D. and S.L. Klein, *Immunological mechanisms mediating hantavirus*
591 *persistence in rodent reservoirs.* PLoS Pathog, 2008. **4**(11): p. e1000172.
592 23. Klingström, J., T. Lindgren, and C. Ahlm, *Sex-dependent differences in plasma cytokine*
593 *responses to hantavirus infection.* Clinical and Vaccine Immunology, 2008. **15**(5): p.
594 885-887.
595 24. Guo, J., et al., *Cytokine response to Hantaan virus infection in patients with hemorrhagic*
596 *fever with renal syndrome.* Journal of medical virology, 2017. **89**(7): p. 1139-1145.

597 25. Khaiboullina, S.F., et al., *Serum cytokine profiles differentiating hemorrhagic fever with*
598 *renal syndrome and hantavirus pulmonary syndrome.* 2017. **8**: p. 567.
599 26. Vapalahti, O., et al., *Hantavirus infections in Europe.* 2003. **3**(10): p. 653-661.
600 27. Shahrara, S., et al., *IL-17–mediated monocyte migration occurs partially through CC*
601 *chemokine ligand 2/monocyte chemoattractant protein-1 induction.* The Journal of
602 Immunology, 2010. **184**(8): p. 4479-4487.
603 28. Zhang, J., et al., *IL-17 promotes scar formation by inducing macrophage infiltration.* The
604 American Journal of Pathology, 2018. **188**(7): p. 1693-1702.
605 29. Jovanovic, D.V., et al., *IL-17 stimulates the production and expression of*
606 *proinflammatory cytokines, IL-β and TNF-α, by human macrophages.* The Journal of
607 Immunology, 1998. **160**(7): p. 3513-3521.
608 30. Kehlen, A., et al., *Expression, modulation and signalling of IL-17 receptor in fibroblast-*
609 *like synoviocytes of patients with rheumatoid arthritis.* Clinical & Experimental
610 Immunology, 2002. **127**(3): p. 539-546.
611 31. Hautala, T., et al., *Young male patients are at elevated risk of developing serious central*
612 *nervous system complications during acute Puumala hantavirus infection.* BMC
613 infectious diseases, 2011. **11**(1): p. 217.
614 32. Huang, X., et al., *Epidemiologic characteristics of haemorrhagic fever with renal*
615 *syndrome in Mainland China from 2006 to 2010.* Western Pacific surveillance and
616 response journal: WPSAR, 2012. **3**(1): p. 12.
617 33. Liang, W., et al., *Mapping the epidemic changes and risks of hemorrhagic fever with*
618 *renal syndrome in Shaanxi Province, China, 2005–2016.* Scientific reports, 2018. **8**(1): p.
619 1-10.
620 34. Braun, N., et al., *Characterization and outcome following Puumala virus infection: a*
621 *retrospective analysis of 75 cases.* Nephrology Dialysis Transplantation, 2010. **25**(9): p.
622 2997-3003.
623 35. Furberg, M., C. Anticona, and B. Schumann, *Post-infectious fatigue following Puumala*
624 *virus infection.* Infectious Diseases, 2019. **51**(7): p. 519-526.
625 36. Hojyo, S., et al., *How COVID-19 induces cytokine storm with high mortality.*
626 Inflammation and Regeneration, 2020. **40**(1): p. 1-7.
627 37. Tisoncik, J.R., et al., *Into the eye of the cytokine storm.* Microbiology and Molecular
628 Biology Reviews, 2012. **76**(1): p. 16-32.
629 38. Wu, H., et al., *Spatial-temporal characteristics and the epidemiology of haemorrhagic*
630 *fever with renal syndrome from 2007 to 2016 in Zhejiang Province, China.* Scientific
631 reports, 2018. **8**(1): p. 1-14.
632 39. Krautkrämer, E., et al., *No gender-related differences in the severity of nephropathia*
633 *epidemica, Germany.* BMC infectious diseases, 2013. **13**(1): p. 457.
634 40. Brundin, P., et al., *Gene expression of estrogen receptors in PBMC from patients with*
635 *Puumala virus infection.* Shock, 2012. **37**(4): p. 355-359.
636 41. Klingström, J., et al., *Sex-dependent differences in plasma cytokine responses to*
637 *hantavirus infection.* 2008. **15**(5): p. 885-887.
638 42. Kuhns, D.B., et al., *Ca2+-dependent production and release of IL-8 in human*
639 *neutrophils.* The Journal of Immunology, 1998. **161**(8): p. 4332-4339.
640 43. Yang, L., et al., *IL-8 mediates a positive loop connecting increased neutrophil*
641 *extracellular traps (NETs) and colorectal cancer liver metastasis.* Journal of Cancer,
642 2020. **11**(15): p. 4384.

643 44. Demeters, P., et al., *Neutrophil activating protein/interleukin-8 stimulates the binding activity of leukocyte adhesion receptor CD11b/CD18 on human neutrophils.* J Exp Med, 1990. **171**: p. 1155.
646 45. Smith, W., et al., *Interleukin-8 induces neutrophil transendothelial migration.* Immunology, 1991. **72**(1): p. 65.
648 46. Harada, A., et al., *Essential involvement of interleukin-8 (IL-8) in acute inflammation.* Journal of leukocyte biology, 1994. **56**(5): p. 559-564.
650 47. Kruger, P., et al., *Neutrophils: between host defence, immune modulation, and tissue injury.* PLoS Pathog, 2015. **11**(3): p. e1004651.
652 48. Strandin, T., et al., *Neutrophil activation in acute hemorrhagic fever with renal syndrome is mediated by hantavirus-infected microvascular endothelial cells.* Frontiers in immunology, 2018. **9**: p. 2098.
655 49. Sadeghi, M., et al., *Cytokine expression during early and late phase of acute Puumala hantavirus infection.* BMC immunology, 2011. **12**(1): p. 65.
657 50. Bernstein, E., et al., *Immune response to influenza vaccination in a large healthy elderly population.* Vaccine, 1999. **17**(1): p. 82-94.
659 51. Wu, D. and S.N. Meydani, *Age-associated changes in immune and inflammatory responses: impact of vitamin E intervention.* Journal of leukocyte biology, 2008. **84**(4): p. 900-914.
662 52. Nagel, J.E., et al., *Decreased proliferation, interleukin 2 synthesis, and interleukin 2 receptor expression are accompanied by decreased mRNA expression in phytohemagglutinin-stimulated cells from elderly donors.* The Journal of clinical investigation, 1988. **81**(4): p. 1096-1102.
666 53. Spolski, R., D. Gromer, and W.J. Leonard, *The γ c family of cytokines: fine-tuning signals from IL-2 and IL-21 in the regulation of the immune response.* F1000Research, 2017. **6**.
668 54. Saavedra, F., et al., *Immune response during hantavirus diseases: implications for immunotherapies and vaccine design.* 2021.
670 55. Brocato, R.L. and J.W.J.V. Hooper, *Progress on the prevention and treatment of hantavirus disease.* 2019. **11**(7): p. 610.
672 56. Strafella, C., et al., *Investigation of genetic variations of IL6 and IL6r as potential prognostic and pharmacogenetics biomarkers: Implications for covid-19 and neuroinflammatory disorders.* 2020. **10**(12): p. 351.
675 57. Fajgenbaum, D.C. and C.H.J.N.E.J.o.M. June, *Cytokine storm.* 2020. **383**(23): p. 2255-2273.
677 58. Keshavarz, M., et al., *Association of polymorphisms in inflammatory cytokines encoding genes with severe cases of influenza A/H1N1 and B in an Iranian population.* 2019. **16**(1): p. 1-10.
680 59. Charbonnel, N., et al., *Immunogenetic factors affecting susceptibility of humans and rodents to hantaviruses and the clinical course of hantaviral disease in humans.* 2014. **6**(5): p. 2214-2241.
683 60. Mustonen, J., et al., *Genetic susceptibility to severe course of nephropathia epidemica caused by Puumala hantavirus.* 1996. **49**(1): p. 217-221.
685 61. Korva, M., et al., *HLA-associated hemorrhagic fever with renal syndrome disease progression in Slovenian patients.* 2011. **18**(9): p. 1435-1440.

687 62. Kanerva, M., et al., *High-producer allele of tumour necrosis factor-alpha is part of the susceptibility MHC haplotype in severe puumala virus-induced nephropathia epidemica.* 1998. **30**(5): p. 532-534.
690 63. Mäkelä, S., et al., *Polymorphism of the cytokine genes in hospitalized patients with Puumala hantavirus infection.* 2001. **16**(7): p. 1368-1373.
692 64. Ter Horst, R., et al., *Host and environmental factors influencing individual human cytokine responses.* 2016. **167**(4): p. 1111-1124. e13.
694 65. Hoffmann, S.C., et al., *Ethnicity greatly influences cytokine gene polymorphism distribution.* 2002. **2**(6): p. 560-567.
696 66. Piasecka, B., et al., *Distinctive roles of age, sex, and genetics in shaping transcriptional variation of human immune responses to microbial challenges.* 2018. **115**(3): p. E488-E497.
699 67. Nakamura, K., Y. Komagiri, and M. Kubokawa, *Interleukin-1β suppresses activity of an inwardly rectifying K+ channel in human renal proximal tubule cells.* The Journal of Physiological Sciences, 2013. **63**(5): p. 377-387.
702 68. Nakamura, K., et al., *Effects of cytokines on activity of an inwardly rectifying K+ channel in cultured human proximal tubule cells.* J Iwate Med Assoc, 2007. **59**: p. 375-385.
704 69. Schmidt, C., et al., *Regulation of renal sodium transporters during severe inflammation.* Journal of the American Society of Nephrology, 2007. **18**(4): p. 1072-1083.
706 70. Tietäväinen, J., et al., *Glucosuria predicts the severity of Puumala hantavirus infection.* Kidney international reports, 2019. **4**(9): p. 1296-1303.
708 71. Koval'skiĭ, I., *Blood lipids and the indicators of lipid peroxidation in patients with hemorrhagic fever with nephrotic syndrome.* Terapevticheskii arkhiv, 1988. **60**(6): p. 82.
710 72. Martynova, E.V., et al., *High triglycerides are associated with low thrombocyte counts and high VEGF in nephropathia epidemica.* Journal of Immunology Research, 2016. **2016**.
713 73. Hardardottir, I., C. Grünfeld, and K.R. Feingold, *Effects of endotoxin and cytokines on lipid metabolism.* Current opinion in lipidology, 1994. **5**(3): p. 207-215.
715 74. Sweep, C.F., et al., *Chronic intraperitoneal infusion of low doses of tumor necrosis factor α in rats induces a reduction in plasma triglyceride levels.* Cytokine, 1992. **4**(6): p. 561-567.
718 75. Duewell, P., et al., *NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals.* Nature, 2010. **464**(7293): p. 1357-1361.
720 76. Fotakis, P., et al., *Anti-inflammatory effects of HDL (High-Density Lipoprotein) in macrophages predominate over proinflammatory effects in atherosclerotic plaques.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2019. **39**(12): p. e253-e272.

723

724

725