1 **Diminished coagulation capacity assessed by calibrated automated thrombography**

2 **during acute Puumala hantavirus infection**

3 Running head: Decreased endogenous thrombin potential in hantavirus infection

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5 Sirpa M. Koskela[1*], Lotta Joutsi-Korhonen[2], Satu M. Mäkelä[1, 3], Heini Huhtala[4], Antti I. Vaheri[5],

6 Ilkka Pörsti[1, 3], Jukka T. Mustonen[1, 3] and Outi K. Laine[1, 3]

7 [1] Department of Internal Medicine, Tampere University Hospital, PO Box 2000, 33521 Tampere, Finland

8 [2] Coagulation Disorders Unit, Clinical Chemistry, HUSLAB Laboratory Services, Helsinki University

9 Hospital, PO Box 372, 00029 Helsinki, Finland

10 [3] Faculty of Medicine and Life Sciences, University of Tampere, 33014 Tampere, Finland

11 [4] Faculty of Social Sciences, University of Tampere, 33014 Tampere, Finland

12 [5] Department of Virology, Faculty of Medicine, University of Helsinki, PO Box 21, 00014 Helsinki, Finland

13 *Corresponding author: Department of Internal Medicine, Tampere University Hospital, PO Box 2000,

14 33521 Tampere, Finland. E-mail: Koskela.Sirpa.M@student.uta.fi

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22 Conflicts of interests: none.

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1

24   **Abstract**

25   **Objectives**

26   Coagulation abnormalities are associated with Puumala virus-induced hemorrhagic fever with renal

27   syndrome (PUUV-HFRS). We evaluated the coagulation capacity of plasma during acute PUUV-HFRS by

28   measuring thrombin generation using calibrated automated thrombography (CAT®).

29   **Material and Methods**

30   The study cohort comprised of 27 prospectively collected, consecutive, hospital-treated patients with acute

31   PUUV infection. Blood samples were drawn in the acute phase and at the control visit approximately 5

32   weeks later. To evaluate thrombin generation, the lag time of initiation, endogenous thrombin potential

33   (ETP), and peak and time to peak thrombin concentration were assessed by CAT® in platelet poor plasma

34   without corn trypsin inhibitor. Plasma levels of D-dimer, fibrinogen and prothrombin fragments (F1+2) were

35   also evaluated.

36   **Results**

37   When the acute phase was compared with the control phase, ETP was decreased (median 1154 nM/min,

38   range 67-1785 vs. median 1385 nM/min, range 670-1970; $p<0.001$), while the lag time was prolonged

39   (median 3.8 minutes, range 2.1-7.7 vs. median 2.9 minutes, range 2.0-4.1; $p<0.001$). Low ETP correlated

40   with low peak thrombin concentration ($r=0.833$, $p<0.001$). Prolonged time to peak associated with the lag

41   time ($r=0.78$, $p<0.001$). ETP was associated with thrombocytopenia ($r=0.472$, $p=0.015$) and weakly with

42   fibrinogen level ($r=0.386$, $p=0.047$). The measured CAT® parameters did not associate with D-dimer and

43   F1+2 levels.

44   **Conclusions**

45   Decreased ETP together with low peak and prolonged lag time indicate decreased plasma potential for

46   thrombin generation *in vitro*. Together with low platelet count and enhanced fibrinolysis this further refers to

47   altered blood coagulation and increased propensity toward bleeding in acute PUUV-HFRS.

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Keywords: coagulation; Calibrated automated thrombography; thrombin; hantavirus; platelet; fibrinolysis

Abbreviations: ADAMTS13, a thrombospondin type 1 domain; APTT, activated partial thromboplastin time; AT, antithrombin; CAT®, calibrated automated thrombography; CRP, C-reactive protein; ETP, endogenous thrombin potential; F1+2, prothrombin fragments; HCPS, hantavirus cardiopulmonary syndrome; HFRS, hemorrhagic fever with renal syndrome; LT, lag time; PC, protein C; PPP, platelet poor plasma; PS, protein S free antigen; PT, prothrombin time; PUUV, Puumala virus; TAFI, thrombin activatable fibrinolysis inhibitor; TF, tissue factor; TG, thrombin generation; tPA, tissue plasminogen activator; TT, thrombin time; tt Peak, time to peak.

Introduction

Hantaviruses are the cause of two disease entities, hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus cardiopulmonary syndrome (HCPS) in North and South America. Puumala hantavirus (PUUV) causes mild HFRS, also called nephropathia epidemica in Europe [1, 2]. PUUV-HFRS is characterized by thrombocytopenia and coagulation abnormalities, acute kidney injury and capillary leakage [1-3]. Petechiae, epistaxis, hematuria and conjunctival bleedings are common [1]. Hemorrhagic gastropathy is observed in all PUUV-HFRS patients in gastroscopy [4]. Severe and fatal hemorrhages of pituitary gland, kidneys, heart, liver, lungs and peritoneal cavity have been described [5, 6]. The risk for cardiovascular disease has also been linked with PUUV-HFRS [7]. Disseminated intravascular coagulation has been encountered in severe cases [8, 9].

Hantaviruses target vascular endothelial cells via ß3 integrin receptor and adhere quiescent platelets to the endothelium, thus contributing to vascular permeability and thrombocytopenia [10]. Through interactions with hantavirus, platelets and endothelium, alterations in the coagulation system occur. Previous studies imply enhanced thrombin formation, as evaluated by shortened prothrombin time (PT) and thrombin time (TT), and overall increase in prothrombin fragments 1+2 (F1+2), and decreased levels of natural anticoagulants, antithrombin (AT), protein C (PC) and protein S free antigen (PS) [8, 11]. A study with PUUV infected human umbilical vein endothelial cells suggests increased tissue factor (TF) activity [12]. Fibrinolysis is activated as indicated by increased concentrations of fibrin degradation products, D-dimer and tissue plasminogen activator (tPA) [8, 13]. Platelet ligands are altered, and ADAMTS13 activity is decreased [14].

Thrombin is the key enzyme during coagulation leading to the conversion of fibrinogen to fibrin and clot formation. Thrombin generation (TG) assays are useful indicators of the overall plasma coagulability, in contrast to the conventional coagulation tests that mainly assess individual factors or a part of the coagulation pathway. Calibrated automated thrombography (CAT®) measures *in vitro* TG of plasma by continuous cleavage of a fluorogenic substrate, thus expressing the overall haemostatic potential [15]. CAT®

82   is applied in research of vascular thrombosis, bleeding disorders and monitoring of anticoagulant treatment

83   [16]. To our knowledge, studies on TG by CAT® in hantavirus infections are yet lacking.

84   Both hemorrhagic and thrombotic events have been associated with PUUV-HFRS, but the underlying

85   mechanisms of alterations in coagulation system are not well defined. Therefore, we aimed to evaluate the

86   plasma coagulation capacity in PUUV-infected patients by measuring TG by CAT®. The goal was to

87   describe how TG is altered during the acute phase of infection, and further determine the possible hypo- or

88   hypercoagulability associated with hantavirus infection. We also sought to investigate the possible

89   associations of CAT® assay with the tests applied to measure thrombin formation and fibrinolysis in clinical

90   practice and variables depicting disease severity of acute PUUV-HFRS.

91   **Material and Methods**

92   *Ethics statement*

93   All patients were recruited and enrolled after providing a written informed consent. The study protocol was

94   approved by the Ethics Committee of Tampere University Hospital. The study was conducted according to

95   the principles expressed in the Declaration of Helsinki.

96   *Patients*

97   The study was carried out in Tampere University Hospital, University of Tampere, Helsinki University

98   Hospital and University of Helsinki. All patients came from the Pirkanmaa area and were hospitalized at

99   Tampere University Hospital due to serologically confirmed acute PUUV-HFRS [17] during the period from

100   October 2010 to February 2014.

101   Twenty-seven prospectively collected, consecutive patients (17 males) with acute PUUV-HFRS were

102   included. Their median age was 50 years (range 21-67 years). None of the subjects used anticoagulant or

103   immunosuppressive therapy. Two patients used an anti-platelet drug (acetylsalicylic acid).

Clinical and laboratory data

105   The following variables were recorded: the number of days from the onset of fever before the acute-phase

106   study samples were collected, the length of hospital stay (days), signs of bleeding symptoms (yes/no),

107   thromboembolic complications (yes/no), need for transient hemodialysis treatment (yes/no), and maximum

108   gain in weight (kg). Complete blood count, plasma C-reactive protein (CRP) and plasma creatinine were

109   measured according to clinical need. The laboratory analyses were carried out at the Laboratory Centre of

110   Pirkanmaa Hospital District using standard methods.

111   *Methods*

112   The study design was longitudinal with two time-points of blood draw for CAT®. The acute phase samples

113   (n=27) were taken median 7 days (range 4-12 days) from the onset of fever. Control samples (n=23) were

114   taken at the follow-up visit, median 43 days (range 38-76 days) from the onset of fever. The blood count was

115   assessed in the acute and control phase of CAT® study days, and the lowest platelet count during the

116   hospital stay was recorded.

117   CAT® analyses and plasma measurements of fibrinogen, F1+2 and D-dimer were carried out in Clinical

118   Chemistry coagulation laboratory (HUSLAB Laboratory Services, Helsinki University Central Hospital,

119   Finland). D-dimer (Tina Quant D-Dimer®, Roche Diagnostics, Mannheim, Germany) and fibrinogen

120   (Multifibren U® Siemens Healthcare Diagnostics) levels were determined according to manufacturer's

121   recommendations. F1+2 were measured by an enzyme immunoassay (Enzygnost® F1+2, monoclonal,

122   Siemens Healthcare Diagnostics). The reference values for D-dimer were ≤ 0.5 mg/l, fibrinogen 1.7-4.0 g/l

123   and F1+2 69-229 pmol/l.

124   *Measurement of thrombin generation by CAT®*

125   TG was measured using CAT® (Diagnostica Stago) with the Stago PPP reagent (tissue factor 5 pM and

126   phospholipids 4 μM) without corn-trypsin inhibitor. The lag time of the initiation of TG (LT, min), the

127   endogenous thrombin potential (ETP; the area under the curve; nM thrombin x time), peak (maximum

128   thrombin concentration, nM) and time to peak (tt Peak, min) were measured and recorded according to the

129   manufacturer's instructions. Blood samples were collected into sodium citrate anticoagulant (3.2%; 109 mM)

130   tubes according to the local sampling protocol as part of hospital routine, and centrifuged (at 2500 g for 15

131   min). The PPP was separated within 2 hours and stored at -80 °C before analysis.

132   *Statistics*

133   All continuous, skewed variables were determined as medians and ranges. The associations between TG

134   markers and clinical and laboratory variables were evaluated for continuous data by Spearman rank

135   correlation coefficient. To analyze the changes between the acute and control phase, Wilcoxon-signed rank

136   test was used for pairwise comparisons. The level of significance was set at p value 0.05 (2-tailed). Statistical

137   analyses were performed with IBM SPSS Statistics for Windows version 22.0 (Armonk, NY, USA).

138   **Results**

139   *Clinical and laboratory findings*

140   All 27 patients suffered from clinically typical and serologically confirmed PUUV-HFRS [17]. The clinical

141   and laboratory findings of the patients are shown in Table 1. Mild bleedings were reported in eight patients

142   including nasal and conjunctival hemorrhages, petechiae, hemoptysis and melena. There were no

143   thromboembolic events recorded. None of the patients needed transient hemodialysis treatment.

144   The acute phase median platelet count was 68 x 10^9/l (range 8-222 x 10^9/l), CRP 57 mg/ml (10-178 mg/ml),

145   hemoglobin 139 g/l (120-177 g/l), hematocrit 0.40 (0.34-0.49) and creatinine 126 μmol/l (52-699 μmol/l).

146   Twenty-four out of 27 patients were thrombocytopenic (lowest platelet count <150 x 10^9/l).

147   *Thrombin generation by CAT®*

148   When compared with the control phase, ETP was diminished by 16% (1154 nM/min, 67-1785 nM/min vs.

149   1385 nM/min, 670-1970 nM/min; p<0.001). Additionally, tt peak was prolonged (7.3 minutes, 4.8-14.9

150   minutes vs. 5.9 minutes, 4.3-9.8 minutes; p=0.012). Peak thrombin concentration was lowered (204 nM, 5.6-

151   293 nM vs. 243 nM, 106-331 nM; p=0.008), and LT was prolonged (3.8 minutes, 2.1-7.7 minutes vs. 2.9

152   minutes, 2.0-4.1 minutes; p<0.001) in the acute phase. Accordingly, ETP correlated with peak thrombin

153 concentration (r=0.833, p<0.001; Fig. 1A). Tt peak associated with the LT (r=0.78, p<0.001; Fig. 1B). An

154 inverse correlation was observed between peak and tt peak (r= -0.54, p=0.004).

155 *Associations of CAT® parameters with variables depicting clinical disease*

156 In the acute phase, ETP associated with the platelet count measured in the CAT® study day sample (r=0.472,

157 p=0.015; Fig. 1C), and with the lowest platelet count measured during the hospital stay (r=0.402, p=0.038).

158 Similarly, peak thrombin concentration associated with the platelet count of the CAT® study day (r=0.554,

159 p=0.003), and with the lowest platelet count measured during the hospital stay (r=0.462, p=0.015). Prolonged

160 LT and low ETP associated with increased fibrinogen level measured in the acute phase (r=0.511, p=0.006

161 and r=0.386, p=0.047, respectively; Fig. 1D). The fibrinogen level was acutely increased compared with the

162 control phase (median 4.2, range 2.2-9.6 g/l and median 3.4 g/l, range 2.6-4.9 g/l, respectively; p=0.005).

163 D-dimer was increased in the acute phase (2.8 mg/l, 0.6-34 mg/l vs. 0.4 mg/l, 0.2-1.1 mg/l; p<0.001).

164 D-dimer associated strongly with F1+2 (r=0.843, p<0.001). F1+2 was increased acutely (704 pmol/l, 284-

165 1875 pmol/l vs. 263 pmol/l, 118-556 pmol/l; p<0.001). ETP did not associate with D-dimer and F1+2

166 (r=-0.079, p=0.695 and r=-0.164, p=0.415, respectively). There were no associations between peak and D-

167 dimer and F1+2 (r=-0.030, p=0.882 and r=-0.162, p=0.418, respectively).

168 Clinical variables depicting the disease severity, i.e. maximum leukocyte count, maximum plasma creatinine,

169 CRP level and length of hospital stay were not associated with CAT® parameters (data not shown).

170 Furthermore, reduced ETP was not associated with bleedings (data not shown).

171 **Discussion**

172 The primary aim of this study was to investigate whether thrombin generation (TG), measured using

173 calibrated automated thrombography (CAT®) assay, is altered during acute Puumala virus -induced

174 hemorrhagic fever with renal syndrome (PUUV-HFRS). In addition, we sought to elucidate the underlying

175 coagulation mechanisms predisposing to bleeding and thrombosis. The main findings were reduced

176 endogenous thrombin potential (ETP) and peak thrombin concentration suggesting diminished plasma

177 potential for TG during acute PUUV-HFRS. Prolonged lag time (LT) indicating slower initiation of burst,

178   and extended time to reach the peak representing the velocity of TG, further support the finding of

179   hypocoagulability. Decreased platelet count, one of the clinical characteristics of hantavirus infection, was

180   found to associate with low ETP and low peak thrombin concentration. We did not find statistically

181   significant associations between prothrombin fragments F1+2 (F1+2), D-dimer and CAT® parameters in this

182   study population.

183   Previous studies imply enhanced TG in acute PUUV-HFRS on the basis of increased prothrombin fragments

184   F1+2 generated during conversion of prothrombin to thrombin, and an increased level of fibrin degradation

185   product D-dimer [8, 9, 11]. High levels of circulating F1+2 and D-dimer observed in this study confirm

186   previous findings. *In vivo* TG parameters, F1+2 and D-dimer, strongly depend on the amount of tissue factor

187   (TF) and thrombomodulin present on vascular endothelial cells [16, 18]. *In vivo* TG is also ongoing in the

188   microparticles released during infection [19, 20]. Data suggests increased TF in endothelial cells in the acute

189   phase of PUUV-HFRS [12]. On the other hand, the *in vitro* thrombin potential assessed by CAT®

190   determines how thrombin can be generated by plasma containing a defined amount of TG trigger. Thus, it

191   measures the haemostatic balance of plasma clotting factors and inhibitors independently of the procoagulant

192   and inhibitory drivers released by the endothelium [18]. The difference between *in vivo* and *in vitro* TG is

193   well reflected in consumption coagulopathy, a condition where indicators of ongoing coagulation are

194   increased, but plasma potential of TG is decreased [21].

195   A previous study indicates slightly prolonged prothrombin time (PT), prolonged activated partial

196   thromboplastin time (APTT), and shortened thrombin time (TT) during acute PUUV-HFRS [8, 18]. These *in*

197   *vitro* coagulation tests cannot detect the *in vivo* contribution of endothelial cells and shear stress of blood

198   flow on local clot formation and fibrinolysis. Traditional coagulation tests APTT and PT assess the time to

199   the initiation of clot formation and thus they do not entirely reflect the hemostatic balance in acutely ill

200   patients [22]. These plasma clotting assays are considered to reflect the LT phase of TG in CAT® assay [22].

201   Our observation of prolonged LT indicating slower initiation of TG is in line with the previous findings of

202   the coagulation tests APTT and PT. As the data concerning *in vitro* coagulation tests is lacking in the current

203   study population, the direct comparison with CAT® parameters is not possible.

204 Fibrinolysis is increased, as indicated by high D-dimer and endothelial cell tissue plasminogen activator

205 (tPA) levels, during PUUV infection [8, 11-13]. The plasminogen activator inhibitor 1 (PAI-1) level is not

206 altered in the acute phase [13]. Thrombomodulin-associated thrombin activates the thrombin activatable

207 fibrinolysis inhibitor (TAFI), which downregulates fibrinolysis. It can be speculated that low ETP may

208 contribute to decreased TAFI and thus increased fibrinolysis and bleeding tendency [23]. Diminished TG in

209 PUUV-HFRS together with excessive fibrinolysis also resembles the data obtained in another hemorrhagic

210 fever, dengue virus infection [24, 25]. In dengue fever the bleeding complications have been shown to

211 associate with reduced thrombin formation along with thrombocytopenia and enhanced fibrinolysis [26]. We

212 did not find an association between low ETP and bleedings, although mild bleedings were reported in one

213 third of the patients.

214 We are aware of the relatively small sample size of the study. Yet, the associations were statistically

215 significant, even if the number of clinical events remained minor. The levels of coagulation factors were not

216 available and individual acquired or inherited factors affecting hemostasis could not be assessed. Two

217 patients used aspirin, a platelet antagonist that inhibits platelet aggregation and thrombin formation. As TG

218 was assessed in platelet poor plasma it is unlikely that aspirin, attached to the minor amount of residual

219 platelets, could affect these CAT® results.

220 Ongoing *in vivo* coagulation may result in consumption of platelets and coagulation factors during acute

221 PUUV-infection. Correlation of low platelet count with low ETP and low peak may imply thrombin

222 activation and consumption of platelets, as thrombopoiesis is shown to be active during acute PUUV-HFRS

223 [20]. Natural anticoagulants, protein C and protein S free antigen and antithrombin are also found to be

224 decreased in the acute phase of PUUV-HFRS [8]. Increased TF expression on the endothelial cells and

225 microparticle release might result in consumption of platelets and clotting factors resulting in lower ETP. All

226 of these findings are supported by the previous reports [8, 11, 20].

227 In conclusion, in this study we found decreased *in vitro* TG measured by CAT® in acute PUUV infection.

228 Together with thrombocytopenia, increased fibrinolysis and signs of enhanced TG *in vivo* this data suggests

229 a mild to moderate consumption coagulopathy during acute PUUV-HFRS. The CAT® results of plasma

230 hypocoagulobility support previous findings of impaired hemostasis during acute PUUV-HFRS. Larger

231 future studies might further clarify the role of coagulation in the pathogenesis of HFRS.

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300   Figure Legend

301

302   Figure 1. Scatter plots illustrating the correlation between endogenous thrombin potential and peak thrombin

303   concentration (Fig.1A), lag time and time to peak thrombin concentration (Fig. 1B), endogenous thrombin

304   potential and simultaneous platelet count (Fig. 1C) and endogenous thrombin potential and plasma

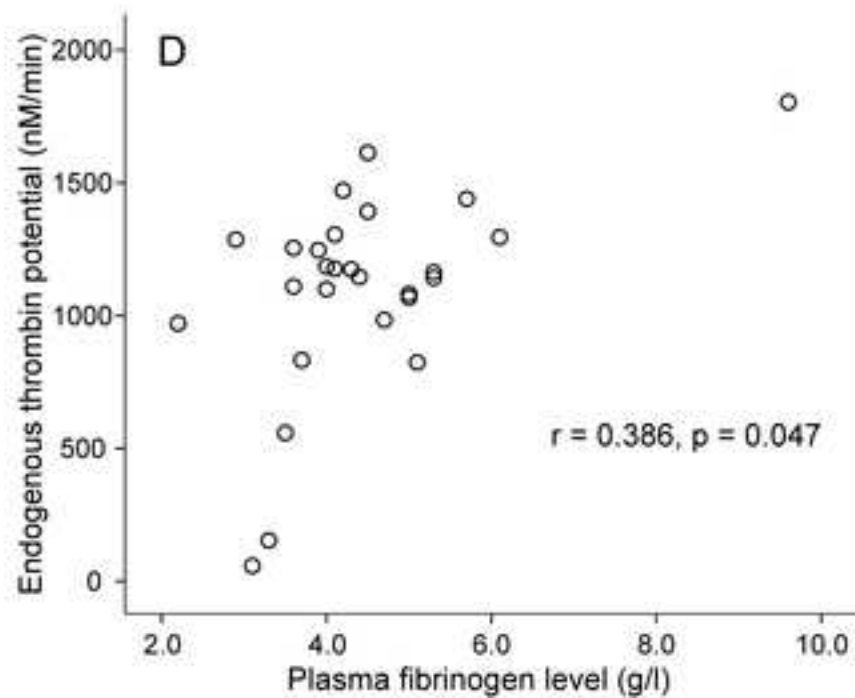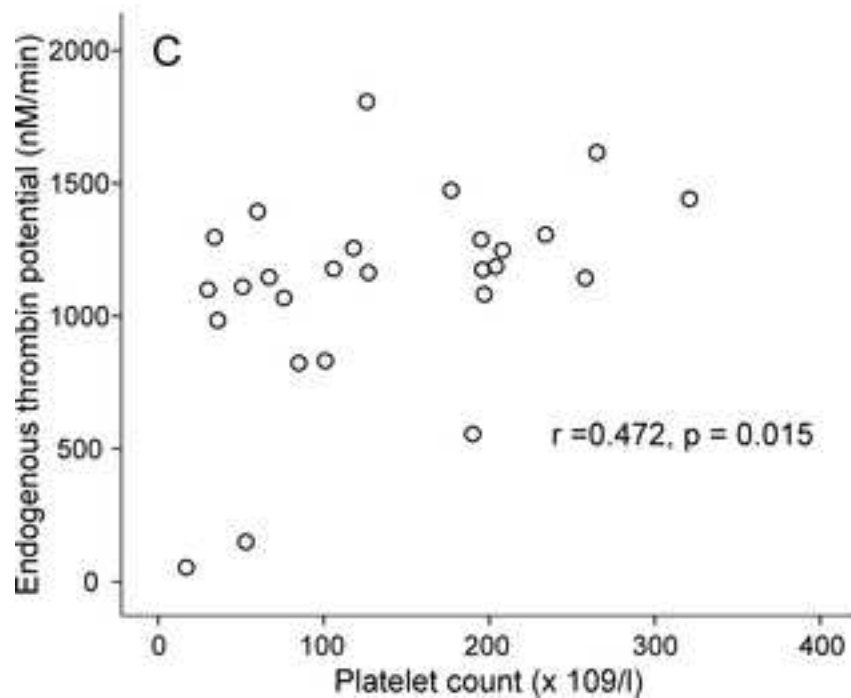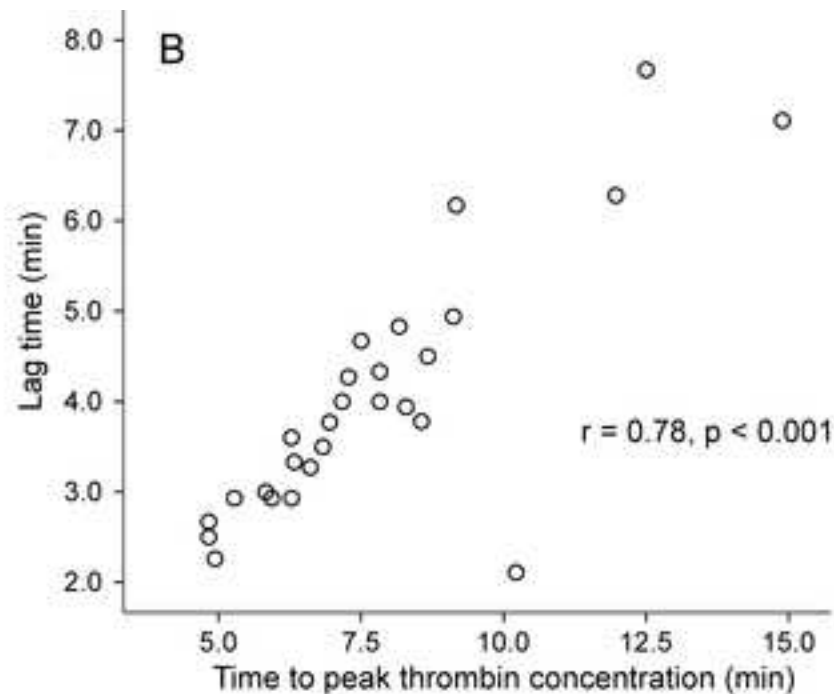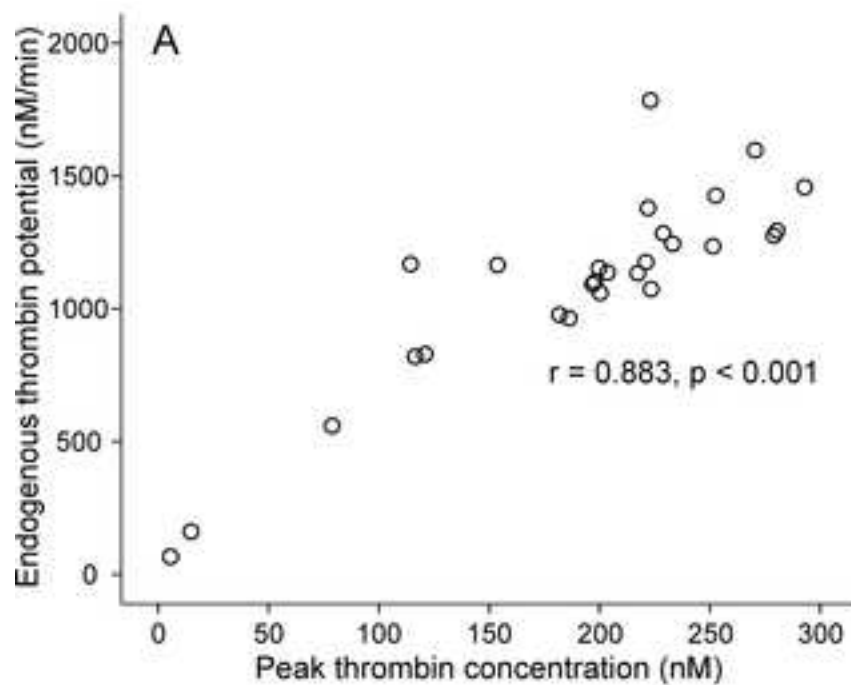305   fibrinogen level (Fig. 1D) during acute PUUV infection.

306

Table 1. The clinical and laboratory findings during hospital care in 27 patients with acute Puumala

hantavirus infection.

Clinical or laboratory variable	Median	Range
Days from the onset of illness[a]	7	4-12
Length of hospital stay (days)	7	3-12
Body mass index (kg/m^2)	26.6	22.3-36.8
Change in weight (kg)[b]	3.8	0.5-11.3
Systolic BP min (mmHg)	108	80-135
Diastolic BP min (mmHg)	67	55-83
Creatinine max (μmol/l)	268	71-983
Leukocyte count max (x 10^9/l)	10.7	4-45
Hemoglobin max (g/l)	155	122-214
Hematocrit max	0.43	0.37-0.60
Platelet count min (x 10^9/l)	60	5-150
CRP max (mg/ml)	79	21-244

Abbreviations: min=minimum, max=maximum, BP=blood pressure.

Reference values: hematocrit 0.35-0.50 for men and 0.35-0.46 for women, platelet count 150-360, leukocyte count 3.4-8.2 x 10^9/l, CRP < 10 mg/ml, creatinine < 105 μmol/l for men and < 95 μmol/l for women.

[a] The number of days of fever before the first study samples were obtained.

[b] Reflects fluid accumulation in the oliguric phase of PUUV-HFRS.