

# Elevated thrombopoietin and platelet indices confirm active thrombopoiesis but fail to predict clinical severity of puumala hantavirus infection

Outi Laine, MD, PhD<sup>a,b,\*</sup>, Lotta Joutsu-Korhonen, MD, PhD<sup>c</sup>, Riitta Lassila, MD<sup>d</sup>, Heini Huhtala, MSc<sup>e</sup>, Antti Vaheri, MD<sup>f</sup>, Satu Mäkelä, MD, PhD<sup>a</sup>, Jukka Mustonen, MD<sup>a</sup>

## Abstract

We evaluated the mechanisms of thrombocytopenia and procoagulant changes in relation with clinical variables in a cohort of patients with acute hantavirus disease.

Blood samples of 33 prospectively recruited, consecutive, hospitalized patients with acute Puumala virus-induced hemorrhagic fever with renal syndrome (HFRS) were collected acutely and at the recovery visit (control). Serum thrombopoietin (TPO) and activity of plasma microparticles (MPs) from various cell sources were measured with enzyme-linked immunosorbent assay-based methods. The results were related to data on platelet indices and functions, coagulation variables, and clinical disease.

Serum TPO was nearly 4-fold higher acutely compared with the control (median 207 pg/mL, range 56–1258 pg/mL vs. median 58 pg/mL, range 11–241 pg/mL,  $P < 0.001$ ) and coincided with high mean platelet volume (MPV) and immature platelet fraction (IPF%). Prothrombin fragments and D-dimer were high acutely compared with the control (F1 + 2 median 704 pmol/L, range 284–1875 pmol/L vs. median 249 pmol/L, range 118–556 pmol/L,  $P < 0.001$ ; D-dimer median 2.8 mg/L, range 0.6–34.0 mg/L vs. median 0.4 mg/L, range 0.2–1.1 mg/L,  $P < 0.001$ ), and associated with low platelet count and severe acute kidney injury (AKI). MPs' procoagulant activity was high acutely only among patients with mild AKI (plasma creatinine below the median at the time of the measurement).

Upregulated TPO together with high MPV and IPF% confirm active thrombopoiesis, but do not predict severity of HFRS. Simultaneously, elevated prothrombin fragments and D-dimer suggest increased consumption of platelets in patients with severe AKI. Activity of platelet-derived MPs in HFRS should be studied with flow cytometry in a larger cohort of patients.

**Abbreviations:** AKI = acute kidney injury, ALT = alanine aminotransferase, CRP = C-reactive protein, HCPS = hantavirus cardiopulmonary syndrome, HFRS = hemorrhagic fever with renal syndrome, HIV = human immunodeficiency virus, IL-6 = interleukin 6, IPF% = immature platelet fraction %, MP = microparticle, MPV = mean platelet volume, PFA-100 = platelet factor analyzer 100, PUUV = Puumala virus, STAT = signal transducers and activators of transcription, TF = tissue factor, TGFβ = transforming growth factor β, TPO = thrombopoietin.

**Keywords:** coagulation, hantavirus, kidney, microparticle, platelet, platelet indices, thrombopoietin

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<sup>a</sup> Department of Internal Medicine, Tampere University Hospital, <sup>b</sup> School of Medicine, University of Tampere, Tampere, <sup>c</sup> Coagulation Disorders Unit, Clinical Chemistry, HUSLAB Laboratory Services, Helsinki University Hospital, <sup>d</sup> Coagulation Disorders Unit, Department of Hematology, Comprehensive Cancer Center, Helsinki University, and Helsinki University Hospital, Helsinki, <sup>e</sup> School of Health Sciences, University of Tampere, Tampere, <sup>f</sup> Department of Virology, Faculty of Medicine, University of Helsinki, Helsinki.

\* Correspondence: Outi Laine, Department of Internal Medicine, Tampere University Hospital, PO Box 2000, 33521 Tampere, Finland (e-mail: outi.laine@uta.fi).

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## 1. Introduction

Hantaviruses are enveloped RNA viruses belonging to the family of *Bunyaviridae*. In humans, they give rise to a spectrum of illnesses called hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. HCPS carries a mortality rate of 40%, whereas the mortality in HFRS is lower (0.4%–15%), but the incidence of HFRS is substantially higher. Classically, HFRS occurs in 5 distinct phases: febrile, hypotensive, oliguric, polyuric, and convalescent. Capillary leakage, thrombocytopenia, and acute kidney injury (AKI) are the key elements in the pathogenesis of HFRS. Specific treatment is not available for hantavirus infection.<sup>[1]</sup>

Low platelet count is a hallmark of hantavirus disease, but the severity of bleeding symptoms varies according to the hantavirus species. Signs of bleeding can be noted in up to one-third of the patients infected with the most common European hantavirus, Puumala virus (PUUV). Hemorrhage has been documented in various organs in fatal cases,<sup>[2]</sup> and mild mucocutaneous involvement has been reported in general. However, thromboembolic complications and increased risk of acute myocardial infarction and stroke have been reported to associate with PUUV-induced HFRS in Swedish patients.<sup>[3,4]</sup> The individual balance between enhanced and hampered coagulation prevails and remains challenging.

Recent studies support the idea of platelet contribution to disease progression and binding to fibrin-covered endothelial cells as a mechanism leading to the decreased platelet count during acute hantavirus infection.<sup>[11]</sup> Upregulated tissue factor (TF) expression on the infected endothelial cells is suggested to induce increased thrombin formation noted during PUUV infection.<sup>[5,6]</sup> Compatibly with endothelial cell activation, elevated level of tissue plasminogen activator and D-dimer have both been observed.<sup>[6,7]</sup> Findings of enhanced platelet ligands are in concordance with those of increased platelet activation.<sup>[8,9]</sup> Large mean platelet volume (MPV) and immature platelet fraction % (IPF%) together with elevated serum thrombopoietin (TPO) level imply active thrombopoiesis.<sup>[9,10]</sup> Despite increased platelet production the aggregation is, however, impaired.<sup>[10]</sup>

We here studied serum TPO level and the procoagulant activity of plasma microparticles (MPs) in 33 Finnish patients who were treated in hospital for acute PUUV infection. We analyzed the results in relation with clinical data, platelet function tests in whole blood (platelet function analyzer PFA-100 and Multiplate), and coagulation variables available. Our aim was to study whether active thrombopoiesis could be confirmed during the thrombocytopenia associated with acute hantavirus infection. We also sought to further characterize the procoagulant changes observed *in vitro* and *in vivo*. As the prediction of disease severity is still of utmost importance in the treatment of hantavirus infection, we particularly compared the relationship of our findings with the clinical disease severity.

## 2. Methods

### 2.1. Patients

The study was carried out at Tampere University Hospital and University of Tampere School of Medicine, Finland. All patients came from Pirkanmaa region and were hospitalized because of serologically confirmed<sup>[11]</sup> acute PUUV infection during the period from October 2010 to November 2015. Written informed consent was obtained from all patients, and the Ethics Committee of Tampere University Hospital approved the study protocol.

The study group consisted of 33 prospectively collected consecutive adult patients (22 males), median age 45 years (range 21–67 years). Twenty-nine of the patients also participated in a previous study determining platelet indices and functions during acute PUUV infection.<sup>[10]</sup> Concomitant diseases included arterial hypertension ( $n=5$ ), diabetes mellitus ( $n=3$ ), hypercholesterolemia ( $n=2$ ), coronary heart disease ( $n=2$ ), sleep apnea ( $n=2$ ), and celiac disease ( $n=2$ ). One patient had chronic obstructive pulmonary disease and 1 patient had undergone splenectomy because of hereditary spherocytosis. No patient was under immunosuppression or anticoagulation. Two patients received anti-platelet therapy (acetylsalicylic acid, 100 mg daily).

### 2.2. Clinical and basic laboratory data

The following variables were recorded: smoking (yes/no), the number of days from the onset of fever up to the acute phase sample collection, the lowest diastolic and systolic blood pressure (mmHg), clinical diagnosis of shock (yes/no), signs of bleeds (yes/no), thromboembolic complications (yes/no), infusion of platelets (yes/no), need for transient hemodialysis treatment (yes/no), maximum and minimum daily urinary output (mL), and gain in weight (kg). The last variable reflects fluid retention during the hospital stay in the oliguric phase of the disease. Complete blood

count, C-reactive protein (CRP), creatinine, and alanine aminotransferase (ALT) were measured in plasma samples according to clinical need. The laboratory analyses were carried out at the Laboratory Centre of Pirkanmaa Hospital District using standard methods.

### 2.3. Assessment of serum TPO level and procoagulant activity of plasma MPs

Serum samples to measure TPO level in the acute phase of the disease were collected median 8 days (range 2–13 days) from the beginning of fever. Citrated plasma for the measurement of procoagulant activity of MPs and coagulation variables (plasma fibrinogen, prothrombin fragments F1 + 2, and D-dimer) as well as whole blood for platelet indices (MPV and IPF%) and functions (PFA-100 and Multiplate) were all collected at the same time, median 7 days (range 1–12 days) from the beginning of fever. The control samples were obtained at the recovery visit, median 43 days (range 31–60 days) from the beginning of fever.

For TPO, blood samples were collected and centrifuged at 1700g for 15 minutes, and the serum was frozen at  $-70^{\circ}\text{C}$ . Human Thrombopoietin Immunoassay Quantikine ELISA was applied according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). In healthy volunteers ( $n=38$ ), the mean level of serum TPO is 74.2 pg/mL (ranging from nondetectable to 228 pg/mL), according to the manufacturer. To measure procoagulant activity of MPs in plasma, citrate-anticoagulated (109 nmol/L sodium citrate) blood samples were centrifuged at 2000g for 15 minutes. Plasma was then separated, further centrifuged at 13,000g for 2 minutes, and frozen at  $-70^{\circ}\text{C}$ . Blood sampling and processing of plasma were in accordance with the instructions of ZYMYPHEN MP-Activity ELISA (ANIARA, HYPHEN BioMed, West Chester, OH) that was applied for analyses. The MP-Activity test kit is a functional assay measuring procoagulant activity of MPs derived from various cellular sources.<sup>[12]</sup> In the assay, circulating MPs in the plasma sample bind to Annexin V on the microplate surface and expose their phospholipid surface, which allows prothrombin activation by factor Xa and Va complex in the presence of calcium. The exposed phospholipid concentration reflects the amount of thrombin generation, which is measured using specific thrombin substrate. According to the manufacturer, concentration of MPs in normal plasma is usually  $\leq 5$  nmol/L (expressed as phosphatidylserine equivalent).

### 2.4. Platelet indices and functions, and coagulation variables

Measurement of MPV and IPF% as well as the assessment of platelet functions with PFA-100 and Multiplate is described in detail in our recent article.<sup>[10]</sup>

Fibrinogen was measured with a modification of the Clauss method (Multifibren U, Siemens Healthcare Diagnostics), D-dimer with an immunoturbidimetric assay (Tina-quant D-Dimer, Roche Diagnostics, Mannheim, Germany), and F1+2 with an enzyme immunoassay (Enzygnost F1+2, monoclonal, Siemens Healthcare Diagnostics). The reference values were 1.7 to 4.0 g/L for fibrinogen,  $\leq 0.5$  mg/L for D-dimer, and 69 to 229 pmol/L for F1+2.

### 2.5. Statistics

Since all continuous variables were skewed, medians and ranges were calculated to describe the data. Numbers and percentages

were used for categorical variables. Comparisons between the groups were based on Mann–Whitney *U* test or Kruskal–Wallis test. To evaluate changes between the acute and the recovery phase, Wilcoxon test was used. Relationships between the continuous variables were examined using Spearman rank correlation coefficient. The limit of significance was set at 0.05 (2-tailed). IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY) was used for computation.

### 3. Results

#### 3.1. Clinical and basic laboratory findings

All 33 patients suffered from clinically typical and serologically confirmed<sup>[11]</sup> PUUV infection and were examined and hospitalized in the acute phase of the illness. All patients recovered. Table 1 presents the clinical and basic laboratory findings of the study patients. Eighteen (55%) of the patients were smokers. Ten (30%) of the patients had signs of bleeding tendency, mostly mild mucosal bleeds. No thromboembolic complications were observed by the time of control (recovery phase) visit. Three patients received platelet transfusions. Four (12%) of the patients were in clinical shock at the time of admission (ie, systolic blood pressure <90 mmHg together with clinical symptoms or signs of shock). Two patients needed transient hemodialysis, one of them and another patient were treated in the intensive care unit.

At the time of the acute phase TPO and MP samples, 14 (45%) patients had their platelet count  $\leq 100 \times 10^9$  cells/L (median  $118 \times 10^9$  cells/L, range  $17\text{--}321 \times 10^9$  cells/L). At the same time-point, the median creatinine value of the patients was  $167 \mu\text{mol/L}$  (range  $68\text{--}983 \mu\text{mol/L}$ ). In the control (recovery phase) sample, the platelet count of all patients had recovered and in all patients exceeded the lower limit of reference range  $150 \times 10^9$  cells/L (median  $248 \times 10^9$  cells/L, range  $197\text{--}507 \times 10^9$  cells/L).

#### 3.2. Serum TPO level and its associations with clinical and laboratory variables

Serum TPO level was nearly 4-fold higher acutely compared with the control (Table 2). The finding of elevated serum TPO level in the acute phase of the disease remained when patients were

divided into 2 groups (below or above the median value) based on the platelet count at the time of TPO measurement and the lowest platelet count (Fig. 1). Large MPV and IPF% coincided with elevated serum TPO level (Table 2), and an association was noted between serum TPO level and IPF% ( $r=.44$ ,  $P=.03$ ).

High serum TPO level coinciding still with thrombocytopenia associated with impaired platelet functions studied in whole blood by both PFA-100 (Fig. 2) and Multiplate in the acute phase of the disease. However, closure times studied with PFA-100 were only slightly prolonged acutely compared with the control despite the lowered platelet count (Table 2). Platelet functions studied with Multiplate are described in detail elsewhere.<sup>[10]</sup> High serum TPO associated with impaired aggregation triggered with all 5 agonists (for adrenalin,  $r=-0.69$ ,  $P<0.001$ ; for adenosine diphosphate,  $r=-0.77$ ,  $P<0.001$ ; for arachidonic acid,  $r=-0.67$ ,  $P=0.001$ ; for collagen,  $r=-0.63$ ,  $P=.002$ ; for thrombin receptor-activating peptide,  $r=-0.50$ ,  $P=.03$ ; for ristocetin,  $r=-0.73$ ,  $P<0.001$ ).

Serum TPO level associated with the length of hospital stay ( $r=0.40$ ,  $P=0.04$ ). A negative association prevailed between TPO and minimum hematocrit ( $r=-0.45$ ,  $P=0.02$ ), platelet count at the time of TPO measurement ( $r=-0.71$ ,  $P<0.001$ ), and minimum platelet count ( $r=-0.67$ ,  $P<0.001$ ). Signs of bleeding tendency did not, however, associate with serum TPO level. No association was found between serum acute-phase TPO level and plasma creatinine, minimum or maximum daily urinary output, gain of weight, or need of transient hemodialysis. Minimum systolic or diastolic blood pressure and smoking did not associate with serum TPO level either (data not shown).

#### 3.3. Plasma coagulation variables

D-dimer was 7-fold higher acutely compared with the value measured at control visit in this group of PUUV-infected patients. F1+2 was clearly elevated (Table 2) and it associated with serum TPO level ( $r=0.47$ ,  $P=0.03$ ). Both D-dimer and F1+2 associated with minimum platelet count ( $r=-0.48$ ,  $P=0.01$  and  $r=-0.46$ ,  $P=0.02$ , respectively), plasma creatinine level at the time of the measurement ( $r=0.57$ ,  $P=0.002$  and  $r=0.50$ ,  $P=0.008$ , respectively) and maximum plasma creatinine ( $r=0.54$ ,  $P=0.004$  and  $r=0.42$ ,  $P=0.03$ , respectively).

**Table 1**

**Clinical and basic laboratory findings in 33 patients with acute Puumala hantavirus infection.**

Clinical or Laboratory Variable	Reference Values	Median	Range
Days from onset of illness*		7	1–12
Length of hospital stay, days		7	3–22
Systolic BP min, mmHg		108	60–135
Diastolic BP min, mmHg		67	36–83
Daily urinary output min, mL		800	20–2320
Daily urinary output max, mL		4880	2000–9610
Weight gain, kg†		3.8	0.5–11.3
Hematocrit min	0.35–0.46/0.50 f/m	0.35	0.25–0.41
Platelet count min, $\times 10^9$ cells/L	150–360	42	4–150
Leukocyte count max, $\times 10^9$ cells/L	3.4–8.2	12.1	4.0–45.0
CRP max, mg/mL	<10	74	21–244
Creatinine max, $\mu\text{mol/L}$	<95/<105 f/m	296	71–983
ALT max, U/L	10–45/70 f/m	59	9–2076

ALT=alanine aminotransferase, BP=blood pressure, f=females, m=males, max=maximum, min=minimum.

\* The number of days of fever before the first study sample.

† Gain in weight during hospital stay reflects the amount of fluid accumulating in the body during the oliguric phase.

**Table 2**

**Serum thrombopoietin level, platelet indices, platelet functions studied by PFA-100 and coagulation variables during Puumala hantavirus infection at acute and at the recovery phase.**

Variable (unit)	Acute Value Median (Range)	Recovery Value Median (Range)	P	Reference Values
TPO, pg/mL	207 (56–1258)	58 (11–241)	<0.001	ND-228
MPV, fL	11.0 (9.4–13.1)	10.5 (9.2–12.0)	0.006	9.0–12.0
IPF%	7.4 (1.8–23.8)	2.3 (0.8–5.2)	<0.001	1.0–5.0
COLL/ADR, s	130 (70–301)	109 (87–250)	0.840	82–150
COLL/ADP, s	92 (56–301)	89 (63–131)	0.055	62–100
Fibrinogen, g/L	4.2 (2.2–9.6)	3.4 (2.6–4.9)	0.005	1.7–4.0
F1+2, pmol/L	704 (284–1875)	249 (118–556)	<0.001	69–229
D-dimer, mg/L	2.8 (0.6–34)	0.4 (0.2–1.1)	<0.001	≤0.5

COLL/ADR=collagen/adrenalin agonist, F1+2=plasma prothrombin fragments, IPF%=immature platelet fraction %, MPV=mean platelet volume, ND=non-detectable, s=second, TPO=serum thrombopoietin level.

### 3.4. Procoagulant activity of plasma MPs

There was no difference in the activity of MPs derived from various cellular origins in the acute phase of PUUV infection compared with the recovery phase. No associations between MPs and clinical or laboratory variables determined in this study could be observed. However, in patients with plasma creatinine value below the median of 167  $\mu\text{mol/L}$ , MPs' procoagulant activity was increased acutely compared with the control (median 2.7 nmol/L, range 0.9–5.3 nmol/L vs. median 1.8 nmol/L, range 0.8–3.2 nmol/L,  $P=0.04$ ). Likewise, MPs' activity was increased acutely among patients with maximum plasma creatinine value below the median of 296  $\mu\text{mol/L}$  (median 2.6 nmol/L, range 1.0–5.3 nmol/L vs. median 1.8 nmol/L, range 0.8–3.2 nmol/L,  $P=0.03$ ). MPs' activity in the acute phase of the disease did not differ from the activity observed at the recovery when patients were divided into 2 groups (below or above median value) based on the platelet count at the time of the MPs' activity measurement or the lowest platelet count.

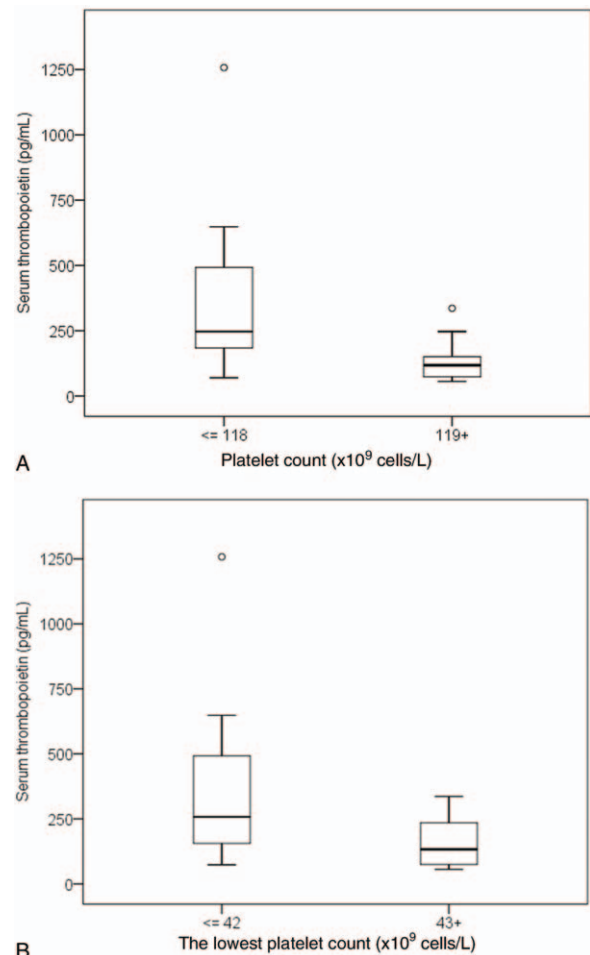
### 3.5. Severely ill patients

Two patients were severely ill and laboratory findings of them are described in Table 3. Patient 1 was a 61-year-old previously smoking male who needed intensive care treatment, and patient 2 was a 28-year-old smoking male with a need of temporary hemodialysis treatment. Both patients had a very low platelet count minimum, high acute TPO and IPF%, and prolonged closure times measured with PFA-100.

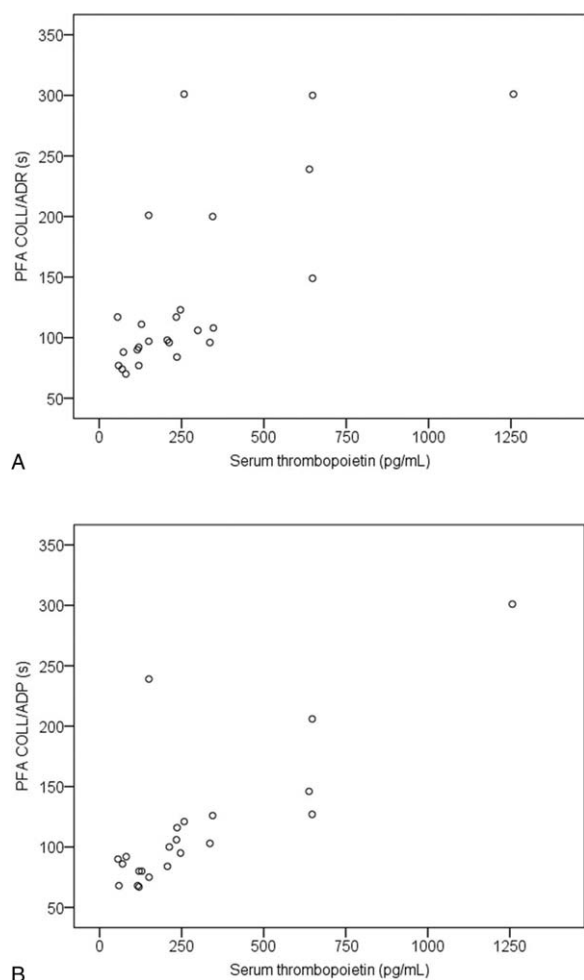
## 4. Discussion

The most evident finding of our study was the 4-fold elevated level of serum TPO during acute PUUV infection compared with the recovery phase. Elevated platelet indices MPV and IPF% imply that bone marrow response to the TPO stimulus is adequate, and decreased platelet count observed during the acute phase of the disease is contributed by factors other than impaired platelet production. The association of high TPO with decreased platelet functions measured both with PFA-100 and Multiplate was observed during thrombocytopenia. It suggests that active thrombopoiesis, though able to provide with clinical hemostasis, is not sufficient to overcome the diminished functional capacity noted in vitro. TPO did not associate with AKI, the major outcome of HFRS. The markedly increased F1+2 and D-dimer further support the idea of platelet consumption in the vasculature by ongoing coagulation and fibrinolysis. Their association with variables reflecting AKI is a novel finding in

Finnish PUUV-patients, but in line with a previous observation of high D-dimer under severe disease condition in Swedish patients.<sup>[3]</sup> We could not detect any increase in MPs' procoagulant activity during acute PUUV infection. That finding, however, needs to be explored in more detail, but MPs have been



**Figure 1.** Serum thrombopoietin level in 33 patients during acute Puumala hantavirus infection. (A) Patients with platelet count at the time of the measurement  $\leq$  or  $>$  the median of  $118 \times 10^9$  cells/L. Serum thrombopoietin median 247 pg/mL, range 70–1258 pg/mL vs. median 118 pg/mL, range 56–336 pg/mL,  $P=.004$ . (B) Patients with the lowest platelet count  $\leq$  or  $>$  the median of  $42 \times 10^9$  cells/L. Serum thrombopoietin median 258 pg/mL, range 74–1258 pg/mL vs. median 133 pg/mL, range 56–336 pg/mL,  $P=0.02$ .



**Figure 2.** Association of serum thrombopoietin level with platelet functions studied with collagen/adrenalin and collagen/adenosine diphosphate agonists of PFA-100. (A)  $r=0.69$ ,  $P<0.001$ . (B)  $r=0.77$ ,  $P<0.001$ .

shown to bind to fibrin surfaces,<sup>[13]</sup> which may be the case during the hantavirus infection.

TPO is a humoral growth factor characterized for its ability to stimulate the production and differentiation of megakaryocytes. TPO is mainly produced in the liver and kidneys, and binding of TPO with its receptor c-mPl on platelet surface initiates intracellular signaling including activation of JAK2/signal transducers and activators of transcription (STAT) pathway. Plasma TPO concentrations vary usually inversely with the platelet count, but mechanisms other than platelet count that affect TPO have also been identified.<sup>[14]</sup>

High plasma concentration of TPO and low platelet count have been reported in patients with Puumala hantavirus, dengue virus, and human immunodeficiency virus (HIV) infection.<sup>[9,15,16]</sup> In HIV-infected patients, c-mPl expression per platelet is elevated, and recombinant human megakaryocytic growth factor reduces the c-mPl expression and restores platelet count to normal level.<sup>[16]</sup> In a macaque model of HIV, transforming growth factor  $\beta$  (TGF $\beta$ ) downregulates TPO, and combined antiretroviral therapy corrects both plasma TGF $\beta$  level and platelet count.<sup>[17]</sup> In severe acute respiratory syndrome induced by a novel coronavirus, TGF $\beta$  inhibits the effect of high TPO on megakaryocytopoiesis.<sup>[18]</sup> Inflammatory mediator interleukin 6 (IL-6),

however, increases TPO production both in vitro and in vivo.<sup>[14]</sup> Our finding of high TPO during acute PUUV infection is in line with a previous observation<sup>[9]</sup> and consistent with both low platelet count and high plasma IL-6 in acute PUUV infection.<sup>[19]</sup> High plasma level of TPO is suggested to predict sepsis severity in the early disease phase.<sup>[20]</sup> We now noted an association between plasma level of TPO and the length of hospital stay, the latter variable describing overall disease severity. However, in this study, TPO had no association with any of the variables reflecting AKI that is the major clinical determinant of hantavirus-induced HFRS.

The inverse association between serum TPO level and platelet functions measured by both PFA-100 and Multiplate was clear, as was the positive association between TPO and IPF%. TPO has been reported to enhance platelet activation during burn injury<sup>[21]</sup> and increase platelet adhesion under flow.<sup>[22]</sup> Moreover, high TPO has a priming effect on platelet aggregation in response to different agonists.<sup>[23]</sup> In this study, the effect of low platelet count on platelet aggregation was not compensated by the simultaneously occurring elevated TPO level and high proportion of young platelets. As major bleeding problems were avoided and no thromboembolic complications were noted, hemostasis appears clinically balanced in spite of laboratory findings reflecting impairment of primary hemostasis.

MPs and exosomes are small membrane vesicles that are released from different cell types by exocytic budding in response to cellular activation or apoptosis. They are now seen as mediators of cell-to-cell communication.<sup>[24]</sup> They express phospholipids and are classically thought to function as procoagulants supporting thrombin formation and facilitating coagulation by factor VII/TF-dependent and -independent pathways. Elevated concentrations of MPs deriving from platelets, endothelial cells, and monocytes have been reported in arterial and venous thrombotic diseases as well as in autoimmune conditions, infectious diseases, hematologic disorders, and cancer. However, the clinical significance of these findings remains controversial.<sup>[25,26]</sup> Flow cytometry is the most widely used method to study MPs.<sup>[27]</sup>

Increased TF expression,<sup>[5]</sup> enhanced thrombin formation,<sup>[6]</sup> and signs of cellular activation and apoptosis<sup>[11]</sup> are all present during acute hantavirus infection, and we presumed to detect higher level of MPs' procoagulant activity during the acute phase of the disease compared with the recovery phase. That was,

**Table 3**

**Laboratory findings of the two patients with severe Puumala hantavirus infection in the acute phase and at the recovery.**

Laboratory Finding	Patient 1	Patient 2
TPO acute/recovery, pg/mL	638/61	648/48
MP acute/recovery, pg/mL	0.9/2.0	5.4/1.0
Plasma creatinine acute, $\mu\text{mol/L}$	574	429
Plasma creatinine max, $\mu\text{mol/L}$	641	756
Platelet count acute, $\times 10^9$ cells/L	85	24
Platelet count min $\times 10^9$ cells/L	4	17
IPF% acute	11.4	13.6
PFA COLL/ADR acute/recovery, s	239/103	300/99
PFA COLL/ADP acute/recovery, s	146/86	206/101

IPF%=immature platelet fraction %, max=maximum, min=minimum, MP=microparticles' procoagulant activity, TPO=serum thrombopoietin level, PFA COLL/ADR/ADP=platelet function analyzer 100 collagen/adrenalin/adenosine diphosphate agonist.

however, the finding only among patients with mild kidney injury. In this study, increased thrombin formation (F1 + 2) and fibrinolysis (D-dimer) associated with the severity of AKI, and the attachment of platelet-derived MPs to fibrin clots especially in patients with more severe kidney injury could plausibly explain our observation.<sup>[13,28]</sup> Preanalytical measures as well as analytical methods in the detection of MPs' activity are crucial<sup>[29]</sup> and even if the instructions of the ELISA-assay manufacturer were vigorously followed, the issues regarding methodology cannot be completely excluded.<sup>[12]</sup> The sample size of the study was, though not large but probably sufficient, as the difference of serum TPO level in the acute phase of the disease compared with the recovery could be verified. MPs' procoagulant activity did not associate with any of the variables depicting disease severity, and it failed to predict clinical outcome in this group of patients with acute Puumala hantavirus infection.

Plasma level of TPO or MPs' activity could not predict severe hantavirus disease in the 33 patients of this study, but the 2 severely ill patients had particularly high TPO level and percentage of young platelets as well as very clearly impaired platelet functions according to PFA-100 in the acute phase of the disease (Table 3). The finding regarding MPs' activity on these 2 individual patients was inconsistent and may stem from patients' medical history, current treatment as well as the condition itself.

In conclusion, serum TPO is upregulated and the production of platelets is enhanced during acute Puumala hantavirus disease. Serum TPO does not predict disease severity in terms of AKI. Biomarkers of thrombin generation and especially fibrinolysis are high in the absence of clinical thrombosis or major bleedings, and they associate with adverse kidney outcome. As thrombopoiesis is active and the proportion of young platelets is high, measuring the activity of platelet-derived MPs with flow cytometry might clarify the role of MPs in the pathogenesis of HFRS.

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