Increased Thrombopoiesis and Platelet Activation in Hantavirus-Infected Patients

Anne-Marie Connolly-Andersen, Erik Sundberg, Clas Ahlm, Johan Hultdin, Maria Baudin, Johanna Larsson, Eimear Dunne, Dermot Kenny, Tomas Lindahl, Sofia Ramström and Sofie Nilsson

Linköping University Post Print

N.B.: When citing this work, cite the original article.

Original Publication:
Anne-Marie Connolly-Andersen, Erik Sundberg, Clas Ahlm, Johan Hultdin, Maria Baudin, Johanna Larsson, Eimear Dunne, Dermot Kenny, Tomas Lindahl, Sofia Ramström and Sofie Nilsson, Increased Thrombopoiesis and Platelet Activation in Hantavirus-Infected Patients, 2015, Journal of Infectious Diseases, (212), 7, 1061-1069.
http://dx.doi.org/10.1093/infdis/jiv161
http://www.oxfordjournals.org/

Postprint available at: Linköping University Electronic Press
http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-122665
Increased thrombopoiesis and platelet activation in hantavirus infected patients

Anne-Marie Connolly-Andersen¹*, Erik Sundberg¹,²*, Clas Ahlm¹, Johan Hultdin², Maria Baudin¹, Johanna Larsson², Eimear Dunne³, Dermot Kenny³, Tomas L. Lindahl⁴, Sofia Ramström⁴ and Sofie Nilsson⁵

¹Infectious Diseases, Dept. of Clinical Microbiology ²Clinical Chemistry, Dept. of Medical Biosciences, Umeå University, SE-901 85 Umeå, Sweden., ³Clinical Research Centre, Royal College of Surgeons in Ireland, Dublin, Ireland, ⁴Dept. of Clinical and Experimental Medicine, Linköping University, Sweden

*Anne-Marie Connolly-Andersen and Erik Sundberg are co-first authors.

Running title: HFRS platelet production and activation

Word count
Abstract: 200
Text: 2946
Conflict of interest

TL Lindahl is the owner of Diapensia HB. All other authors have no conflict of interest.

Sources of funding

This work was supported by the Medical Faculty of Umeå University and the County Council of Västerbotten (Grant no’s: 216851, 243061, 238461, 321411); and the County Councils of Northern Sweden (Grant no: 296301), and The Heart Foundation of Northern Sweden.

*Corresponding author:

Anne-Marie Connolly-Andersen, PhD

Division of Infectious Diseases

Dept. of Clinical Microbiology

Umeå University

901 85 Umeå

Sweden

Phone number: +46 90 785 09 21

Fax: +46 90 13 30 06

E-mail: Anne-marie.connolly-andersen@climi.umu.se
Abstract

Background: Thrombocytopenia is a common finding during viral hemorrhagic fever, which includes hemorrhagic fever with renal syndrome (HFRS). The two main causes for thrombocytopenia are impaired thrombopoiesis and/or increased peripheral destruction of platelets. In addition, there is an increased intravascular coagulation risk during HFRS, which could be due to platelet activation.

Methods: Thrombopoiesis was determined by quantification of platelet counts, thrombopoietin, immature platelet fraction and mean platelet volume during HFRS. The in vivo platelet activation was determined by quantification of soluble P-selectin (sP-selectin) and glycoprotein VI (sGPVI). The function of circulating platelets was determined by ex vivo stimulation followed by flow cytometry analysis of platelet surface bound fibrinogen and P-selectin exposure. Intravascular coagulation during disease was determined by scoring for disseminated intravascular coagulation (DIC) and recording thromboembolic complications.

Results: The levels of thrombopoietin, immature platelet fraction and mean platelet volume all indicate increased thrombopoiesis during HFRS. Circulating platelets had reduced ex vivo function during disease compared to follow up. Most interestingly, we observed significantly increased in vivo platelet activation in HFRS patients with intravascular coagulation (DIC and thromboembolic complications) as shown by sP-selectin and sGPVI levels.

Conclusion: HFRS patients have increased thrombopoiesis and platelet activation, which contributes to intravascular coagulation.
Key words: Platelets, viral hemorrhagic fever, hemorrhagic fever with renal syndrome, hantavirus, disseminated intravascular coagulation, thrombosis
Introduction

Viral hemorrhagic fevers (VHFs) are characterized by thrombocytopenia, vascular dysfunction and disseminated intravascular coagulation (DIC). Some of the most known VHFs include Ebola (*Filoviridae*), Dengue (*Flaviviridae*), Lassa fever (*Arenaviridae*), Crimean-Congo hemorrhagic fever, Rift Valley Fever and hantaviral disease (*Bunyaviridae*) [1, 2]. Pathogenic hantaviruses cause hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome in the Americas [3]. The European Puumala virus (PUUV) causes HFRS and is considered a mild viral hemorrhagic fever [1]. Thrombocytopenia is one of the main determinants for clinical outcome in hantaviral diseases [4, 5]. The causes of thrombocytopenia during HFRS are largely unknown however the two main causes are impaired thrombopoiesis and/or peripheral platelet destruction [2]. There is a current gap of knowledge regarding platelet regeneration and function, which is crucial for understanding HFRS pathogenesis and increased coagulation risk.

Thrombopoiesis impairment could be due to decreased thrombopoietin (TPO) levels, which is the primary regulator of thrombopoiesis [6]. As far as we know, there is no information on hantavirus infections and thrombopoietin. Although some VHFs impair platelet regeneration by megakaryocyte dysfunction [7, 8], this seems unlikely for hantaviruses since megakaryocyte function and bone marrow count were not affected by hantavirus infection [9, 10]. Increased thrombopoiesis results in an increased immature platelet fraction (IPF), which have a larger mean platelet volume (MPV) and contain increased amounts of RNA (thereby the alternative name reticulated platelet) [11]. Increased IPF and MPV indicate augmented megakaryocyte production of platelets. Our first objective was to determine whether thrombocytopenia could be due to decreased TPO levels, and to quantify IPF and MPV levels as markers of ongoing thrombopoiesis during HFRS.
Peripheral platelet destruction can be caused by platelet activation and consumption, which occurs when platelets adhere to activated endothelial cells or sites of blood vessel injury with exposure of underlying extracellular matrix [2]. This is followed by platelet activation, secretion of storage organelles and aggregation to form thrombi [12]. Platelet activation leads to integrin receptor glycoprotein (GP) IIb/IIIa conformation changes enabling it to bind fibrinogen [13]. Additionally, activation leads to α-granule release resulting in increased platelet surface P-selectin, where it mediates leukocyte binding [2]. The collagen receptor GPVI is specific for platelets and megakaryocytes, and induces platelet activation following stimulation [14]. Soluble levels of the platelet receptors P-selectin and GPVI indicate in vivo platelet activation and can thereby function as an indirect marker for peripheral platelet destruction [14-16]. Previously, the levels of sP-selectin and sGPVI were shown to associate with DIC, acute myocardial infarction (AMI) and stroke [14, 17-19]. Patients with HFRS have increased coagulation risk as shown by the incidence of DIC, AMI and stroke [20, 21]. This indicates that increased peripheral platelet destruction could be one of the causes for thrombocytopenia. Our second objective in this study was therefore to study platelet activation (sP-selectin and sGPVI) during HFRS, specifically in relation to intravascular coagulation (DIC and thromboembolic complications). Furthermore, we aimed to study the function of remaining circulating platelets during HFRS using a novel flow cytometry method.
Materials and Methods

Materials and methods

Study group

Patients (n = 35) were enrolled in the study following verification of PUUV infection. The diagnosis was confirmed by clinical manifestations typical of HFRS followed by detection of IgG and IgM antibodies to PUUV using an immunofluorescence assay [22]. The clinical symptoms and routine laboratory tests were obtained for each patient consecutively. The Regional Ethical Review Board in Umeå approved the study and all patients gave oral and written informed consent.

Peripheral venous blood samples

Platelet poor plasma was obtained by centrifugation of patient blood in vacuum tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA) for 20 minutes at 1500 g. The plasma samples were then stored at -80°C until use for analysis of specified markers. Blood samples for platelet regeneration analysis were drawn into vacuum tubes containing EDTA. All samples were analyzed within 4 hours of collection.

Platelet regeneration

Platelet counts were performed using an automated hematology analyzer, Sysmex XE-2100, upgraded with the IPF Master Software (Sysmex Corporation, Kobe, Japan). MPV and reticulated platelets, measured as an IPF percentage (IPF %), were obtained on the same hematology analyzer as previously described [23, 24].

TPO levels in patient plasma was analyzed using a commercially available ELISA kit (catalog no. DTP00B) according to the instructions of the manufacturer (R&D systems, Minneapolis,
USA). Where TPO values were below the lowest level of detection, the value for detection limit was inserted (31 pg/mL).

Platelet and endothelial activation

Soluble P-selectin levels were quantified using a commercially available ELISA kit according to the instructions of the manufacturer (Cat. No. BBE6, R&D system, Minneapolis, USA). sGPVI levels were measured by a newly developed ELISA, which is described in detail in Supplementary Methods. Von Willebrand Factor (vWF) levels were quantified by ELISA performed with reagents purchased from DAKO (Copenhagen, Denmark). The flow cytometry analysis of ex vivo platelet activation is described in detail in Supplementary Methods.

DIC scoring and thrombosis

The patients were scored into DIC vs. no DIC groups according to a modified score taking into account the impact of infection [20]. The HFRS patients were categorized into groups of “no thrombosis” vs. “thrombosis” depending on whether they had had a radiologically verified thromboembolic event during disease (within 30 days post disease onset (DPDO).

Statistical analysis

Statistical analyses were performed in SPSS for Windows (version 22, IBM, USA). Longitudinal changes in platelet production parameters (platelet count, MPV, IPF, TPO), platelet activation in vivo (sP-selectin and sGPVI plasma levels) and ex vivo (percentage activated platelets) and endothelial activation (vWF plasma levels) were analyzed using generalized estimation equations (GEE) assuming an exchangeable correlation structure between repeated observations. Outcome is presented by pairwise comparisons of means at
different time points with follow-up (at least 31 DPDO) with corresponding standard errors of the mean (SEM). The means for platelet production and platelet activation markers were tested for association using the GEE method for all samples within 16 DPDO. Pairwise comparison of the groups: DIC vs. no DIC and thrombosis vs. no thrombosis, for the platelet production and activation markers were presented within each specified time point using GEE. The association between the maximum level of TPO, IPF, MPV, sP-selectin and sGPVI and the maximum DIC score during HFRS for each patient was determined using Spearman rank correlation coefficient. Only patients with two or more values for each specified analyte were included in this analysis. Wilcoxon-signed rank test was used for pairwise comparison of related variables. The level of significance was set at $P < .05$.

**Results**

**Clinical findings**

A total of 35 patients were included at the Clinic of Infectious Diseases at Umeå University Hospital either after hospitalization or seen by the on-call doctor and later followed as outpatients in this study during October 2007 to May 2013. The demography, clinical characteristics and laboratory values of all patients are shown in Table 1.

**Platelet kinetics**

During the early acute phase of HFRS, all but one patient had a platelet count lower than 150×10^9/L (Figure 1 and Supplementary Figure 1A and 1B). This was followed by intensive platelet production where 31% of the patients had thrombocytosis (> 400×10^9/L) (Supplementary Figure 1C). The platelet count stabilized for most patients after 30 DPDO (Supplementary Figure 1A). Platelet counts during 31-90 compared to > 90 DPDO was not significantly different (data not shown).
Platelet regeneration

The primary regulator of thrombopoiesis, TPO, and ongoing thrombopoiesis markers IPF and MPV were quantified at specified time points in samples from HFRS patients. TPO values peak at the earliest phase of HFRS, and was significantly elevated up to 12 DPDO compared to follow up (> 31 DPDO) (Figure 2A). IPF was significantly higher in all time points up to and including 12 DPDO compared to follow up (Figure 2B). MPV levels were significantly higher during 1-8 DPDO and significantly lower during 13-16 DPDO compared to follow up (Figure 2C). When analyzing the association between platelet count, IPF, MPV and TPO levels in the time points preceding and including platelet count peak (≤ 16 DPDO), all associated significantly with each other (Supplementary Table 1).

Platelet activation

Plasma levels of sP-selectin and sGPVI were analyzed as markers for in vivo platelet activation (Figure 3A and 3B). The kinetics of these two markers were similar to each other. sP-selectin was significantly associated with sGPVI in samples obtained prior to 30 DPDO (data not shown). In addition, levels of sP-selectin and sGPVI associated positively with platelet count and negatively with TPO in samples obtained within 16 DPDO from HFRS patients (Supplementary Table 2).

sP-selectin is also stored in the Weibel-Palade bodies of endothelial cells [25]. vWF levels were quantified as a measure of endothelial Weibel-Palade release (Figure 3A). The kinetics differed with vWF peak preceding sP-selectin peak (Figure 3A). In addition, there was no significant association between sP-selectin and vWF obtained at the same time points during HFRS (data not shown). The maximum level of vWF and sP-selectin associated positively (ρ
= 0.493, \( P = .005 \)), but the maximum level sGPVI did not (data not shown). vWF did not associate with platelet count or TPO levels (Supplementary Table 2).

**Platelet function**

To assess the function of the circulating platelets, we also followed the changes in *ex vivo* platelet activation potential during disease in a subset of the study group (n = 12) by flow cytometry. Generally, the proportion of platelets that bound fibrinogen (Figure 4) or exposed P-selectin on the platelet surface (Figure 5) following agonist stimulation was significantly lower during disease compared to follow up. The percentage of non-stimulated platelets that expressed P-selectin was significantly higher during disease compared to follow up, indicating platelet activation *in vivo* in HFRS patients (Supplementary Table 3). However, addition of the ADP-cleaving enzyme apyrase significantly decreased platelet activation, demonstrating that circulating platelets during HFRS possess auto-activation potential and are therefore not pre-activated *in vivo* to the extent of exhaustion (Supplementary Table 4).

**Platelet regeneration and activation in relation to DIC and thrombosis**

The levels of soluble plasma P-selectin and GPVI were generally higher for patients with DIC and was significantly higher early (Figure 6A) and late (Figure 6B) during disease, respectively. The maximum levels of IPF, MPV and sP-selectin were positively associated with the maximum DIC score for each patient during disease (Supplementary Figure 2). sP-selectin levels were significantly higher in patients with thromboembolic events compared to patients without events (Figure 6C). There were no significant differences between the levels of sGPVI, but the variation was large (data not shown).
Discussion

In our study, we showed increased levels of TPO early during HFRS, which is the primary regulator of thrombopoiesis. This resulted in increased thrombopoiesis as shown by the enhanced levels of IPF and MPV. We thereby show at several levels that thrombopoiesis is increased and functional during HFRS. Increasing sP-selectin and sGPVI levels illustrate platelet activation during hantavirus infection. Most interestingly, we show that the levels of the platelet activation markers sP-selectin and sGPVI were significantly higher in patients with demonstrated intravascular coagulation (DIC and thrombo-embolic complications). The remaining circulating platelets in HFRS patients were dysfunctional as shown by decreased response to ex vivo stimulation compared to follow up.

There are two main causes for thrombocytopenia, either decreased thrombopoiesis and/or increased peripheral destruction [2]. Our clinical study based on consecutive patient sampling establishes that thrombopoiesis is functional and increased during HFRS. We investigated the primary regulator of thrombopoiesis TPO through to the markers for ongoing thrombopoiesis IPF and MPV. Other HF viruses such as Dengue and Junin virus infect and impair megakaryocyte production of platelets [7, 8], but this does not occur for hantaviruses [9, 10]. Though thrombocytopenia characterizes VHFs, different mechanisms precipitate platelet decrease during disease. The increased thrombopoiesis during HFRS observed in our study, indicate peripheral platelet destruction is the likely underlying cause of thrombocytopenia. This can be caused by platelet activation and consumption (e.g. DIC, activated or damaged blood vessels) or by immunological destruction [2]. In our study, the platelet activation markers sP-selectin and sGPVI increase and peak during late HFRS demonstrating in vivo platelet activation and consumption. However, platelet activation does not associate with platelet nadir in our HFRS patients indicating that some other unknown mechanism is
responsible for thrombocytopenia. For example, it is possible that hantaviral binding to platelets induces phagocytosis by macrophages as shown for another member of the Bunyaviridae [26] or sequestration by hantavirus infected endothelial cells [27]. The exact underlying mechanism for thrombocytopenia at disease onset has yet to be clarified for HFRS.

In our study the increased levels of TPO precede the platelet peak and in some cases thrombocytosis in the patients. This substantiates previous observations where TPO elevation induces thrombocytosis in patients with a bacterial or viral infection [28]. Interestingly, the inflammatory cytokine IL-6 was responsible for thrombocytosis in an in vivo study [29]. IL-6 induces hepatic TPO production during inflammation [6]. It seems likely that IL-6 is the cause of increased TPO production and thereby the thrombocytosis observed in our study, since both HFRS disease severity and platelet counts were associated with IL-6 levels in a previous study [30]. Of further note is the finding that thrombocytosis is an independent risk factor for thrombosis in patients that have predisposing risk factors [31, 32]. Whether this is the case for HFRS patients has yet to be shown but it is worth mentioning that in the present study we observed a significantly higher level of TPO in patients with DIC and thromboembolic complications (data not shown). Further studies are warranted to clarify this issue.

Soluble P-selectin is also stored in the Weibel-Palade granules of endothelial cells and released upon activation, therefore the levels of circulating P-selectin is not solely derived from activated platelets [25]. To circumvent this issue in our study, we included vWF as a marker for endothelial activation [33], and we found no significant association between vWF and sP-selectin. In addition, the similar time course of sP-selectin and the specific platelet activation markers sGPVI during HFRS and the highly significant association between these
indicates that most of the sP-selectin observed in our study is derived from platelets. The close correlation between the platelet count and sP-selectin and sGPVI levels raises the issue of whether platelets are activated in the vials following collection of blood samples. The blood samples were treated similarly at all time points, and at follow up the platelet count had normalized, yet here the levels of sP-selectin and sGPVI had decreased. If platelets were activated in the vials in vitro, it would be expected that the levels of sP-selectin and sGPVI would remain high especially as the platelet numbers were higher.

We illustrate a decreased function of the circulating platelets in our HFRS patients compared to follow up. Platelets were stimulated with four different agonists targeting the thrombin receptors PAR1 and PAR4, the collagen receptor GPVI or the ADP receptors P2Y₁ and P2Y₁₂, and the result was similar for all receptor-mediated activation responses. A few early studies found decreased platelet aggregation during HFRS [34, 35]. However, aggregometry as a measure of platelet function is affected by the number of platelets and during severe thrombocytopenia the results become less conclusive [36, 37]. The method used in our study does not have this problem since it is independent of the platelet count in the HFRS patient. Our method therefore yields reliable platelet function results even during severe thrombocytopenia. We also show a slight in vivo pre-activation of circulating platelets as illustrated by the small though significant increase of spontaneous P-selectin expressing platelets during HFRS. Previous studies have highlighted this phenomenon in hanta- and dengue virus infected patients, and suggested it to be an “exhausted platelet syndrome” [38, 39]. However, in our study the circulating platelets retained their ability to enhance the level of fibrinogen binding via autocrine ADP-mediated stimulation early during HFRS. We therefore show that platelets are not pre-activated to the degree of exhaustion as previously speculated.
The HFRS patients in our study with intravascular coagulation (DIC and thromboembolic complications) have significantly increased \textit{in vivo} platelet activation as shown by sP-selectin and sGPVI levels. Furthermore, the peak vWF and sP-selectin levels were positively associated in our study; indicating the magnitude of endothelial activation can determine the level of subsequent platelet activation although these are separated in time. HFRS patients have increased thrombin formation and fibrinolysis [40], and are at risk for intravascular coagulation such as DIC, AMI and stroke [20, 21]. The underlying mechanisms have yet to be elucidated for this risk, however it seems likely that activation of platelets as shown in this study and endothelial cells as shown by us in a previous study [41] are likely contributors.

There are limitations to our study. Our study is based on observational data thereby the associations do not necessarily imply causation. The patients presented at different time points post disease onset therefore we do not have access to data for all time points from all patients. The platelet \textit{ex vivo} studies were performed in a sub-group of the patients, therefore we could not stratify the data for DIC. Finally, only two out of our study group had thrombo-embolic events.

A major strength of our study is that we use four different agonists that are mainly specific for different platelet receptors and analyze platelet activation by two different activation markers.

We show that thrombocytopenia during HFRS is likely due to peripheral platelet destruction. Most interestingly, \textit{in vivo} platelet activation markers are higher in patients with coagulation (DIC and thromboembolic complications), which could partially explain the increased risk for acute myocardial infarction and stroke during HFRS. Markers of platelet production and
activation could be used as clinical guidelines for further treatment of HFRS and decrease the
risk for associated sequelae.
Acknowledgements

We would like to acknowledge personnel at the Clinic of Infectious Diseases at Umeå University Hospital for including patients and obtaining plasma samples. We would like to thank patients for participating thereby enabling this study in the first place. We would also like to thank: Jenny Hernestål Boman and Annette Broberg from Research unit Skellefteå, Department of Public Health and Clinical Medicine, Umeå University for analysis of plasma samples. We would like to thank Elisabeth Grönlund and Elin Arvidsson, Dept. of Medical Biosciences, Umeå University for performing flow cytometry platelet function analysis.

Sources of funding

This work was supported by the Medical Faculty of Umeå University and the County Council of Västerbotten (Grant no’s: 216851, 243061, 238461, 321411), the County Councils of Northern Sweden (Grant no: 296301 ), and The Heart Foundation of Northern Sweden.

Disclosures

TL Lindahl is the owner of Diapensia HB. All other authors have no conflict of interest.


Table 1. Characteristics of hemorrhagic fever with renal syndrome patients *

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (median and IQR)</td>
<td>55 (36-57)</td>
<td>NA</td>
</tr>
<tr>
<td>Sex, n female/male (%)</td>
<td>22/13 (63/37)</td>
<td>NA</td>
</tr>
<tr>
<td>Hospital care, n (%)</td>
<td>29 (83)</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Laboratory data**†

<table>
<thead>
<tr>
<th>Laboratory data†</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min platelet count, 10⁹/L</td>
<td>70 (47-88)</td>
<td>145 - 387</td>
</tr>
<tr>
<td>Max platelet count, 10⁹/L</td>
<td>315 (256-412)</td>
<td>145 - 387</td>
</tr>
<tr>
<td>Thrombocytopenia, n (%)</td>
<td>33 (94)</td>
<td>&lt; 100×10⁹/L</td>
</tr>
<tr>
<td>Severe thrombocytopenia, n (%)</td>
<td>9 (26)</td>
<td>&lt; 50×10⁹/L</td>
</tr>
<tr>
<td>Thrombocytosis, n (%)</td>
<td>11 (31)</td>
<td>&gt; 400×10⁹/L</td>
</tr>
<tr>
<td>Serum max creatinine, µmol/L</td>
<td>170 (106-276)</td>
<td>50-100</td>
</tr>
<tr>
<td>Creatinine, highest fold difference‡</td>
<td>2.2 (1.5-3.2)</td>
<td>NA</td>
</tr>
<tr>
<td>Min serum albumin, g/L</td>
<td>28.5 (24.75-31.25)</td>
<td>36-48</td>
</tr>
<tr>
<td>Albumin, lowest fold difference§</td>
<td>0.62 (0.57-0.72)</td>
<td>NA</td>
</tr>
<tr>
<td>Max lactate dehydrogenase, µkat/L</td>
<td>4.9 (4.4-5.8)</td>
<td>&lt; 4.2</td>
</tr>
<tr>
<td>Max C-reactive protein, mg/L</td>
<td>115 (53-170)</td>
<td>&lt; 3</td>
</tr>
</tbody>
</table>

**Hemostasis data**†

<table>
<thead>
<tr>
<th>Hemostasis data†</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max prothrombin/international normalized ratio (PT/INR)</td>
<td>1.1 (1-1.2)</td>
<td>&lt; 1.2</td>
</tr>
<tr>
<td>Max partial thromboplastin time (PTT), sec</td>
<td>30.9 (29.9-34.3)</td>
<td>23-39</td>
</tr>
<tr>
<td>Min Fibrinogen, g/L</td>
<td>4 (3.2-4.6)</td>
<td>2.0-4.5</td>
</tr>
<tr>
<td>Max D-dimer, mg/L</td>
<td>1.3 (0.7-1.8)</td>
<td>&lt; 0.2</td>
</tr>
</tbody>
</table>

| DIC, yes/no (%)                      | 10/25 (29/71) | NA        |
| Thrombosis, yes/no (%)               | 2/33 (6/94)   | NA        |

*Values obtained within 30 days post disease onset (DPDO) for all study participants (n = 35).
NA = not applicable
† The values are shown as median with interquartile range in brackets apart from where frequencies are indicated.
‡ The creatinine levels at follow up was set as baseline for each patient, and all other creatinine levels were compared against this. The value shown is the highest fold difference observed for the patient within 30 DPDO.
§ The albumin level at follow up was set as baseline for each patient and all other albumin values were standardized against this value. The value shown is the lowest fold difference for the patient within 30 DPDO.
Figure 1. Platelet kinetics in patients with hemorrhagic fever with renal syndrome (HFRS). The estimated marginal means for platelet levels were calculated using the generalized estimating equation (GEE) and error bars indicate the standard error of the mean. The numbers underneath each time point represents the number of study individuals with one or more samples included in that time point. Statistical analysis for each time point was calculated using GEE for each time point versus samples obtained after 31 days post disease onset (shown as #). * $P < .05$; ** $P < .01$; *** $P < .001$. 
Figure 2. Platelet regeneration in patients with hemorrhagic fever with renal syndrome (HFRS). The estimated marginal means calculated for (A) thrombopoietin (TPO), (B) immature platelet fraction (IPF) and (C) the mean platelet volume (MPV) using the generalized estimating equation (GEE) method. The error bars indicate the standard error of the mean. The numbers underneath each time point represents the number of study individuals with one or more samples included in that time point. The GEE method was used to calculate whether the values of TPO, IPF and MPV differed significantly from samples obtained after 31 days post disease onset (shown as #). * P < .05; ** P < .01; *** P < .001.
Figure 3. Platelet activation in patients with hemorrhagic fever with renal syndrome. The estimated marginal means calculated for (A) soluble P-selectin, vWF (control for endothelial activation; grey broken line) and (B) soluble GPVI using the generalized estimating equation (GEE) method. The standard error bars indicate the standard error of the mean. The numbers underneath each time point represents the number of study individuals with one or more samples included in that time point. The GEE method was used to calculate whether the difference between levels of sP-selectin and sGPVI for each time point compared to samples obtained after 31 days post disease onset was significant (shown as #). * $P < .05$; ** $P < .01$; *** $P < .001$. 
Figure 4. Fibrinogen binding following ex vivo activation of platelets from hemorrhagic fever with renal syndrome (HFRS) patients. Platelets were stimulated ex vivo with the agonists ADP (10 µmol/L, A), PAR-1-activating peptide (15 µmol/L, B), PAR-4-activating peptide (400 µmol/L, C) and collagen-related peptide (CRP) (0.5 µg/mL, D). The estimated marginal means were calculated using the generalized estimating equation (GEE) for percentage of activated platelets as measured by flow cytometric analysis of platelet fibrinogen binding relative to the isotype control. Error bars indicate the standard error of the mean. The number of study individuals with one or more samples included in each time point: 1-7 days post disease onset (DPDO) n = 10; 8-14 DPDO n = 5; 15-30 DPDO n = 3 and > 31 DPDO n = 12. The GEE method was used to calculate whether percentage fibrinogen-binding platelets differed between each time point compared to follow up (> 31 DPDO; shown as #). * P < .05; ** P < .01; *** P < .001.
Figure 5. P-selectin exposure following *ex vivo* activation of platelets from hemorrhagic fever with renal syndrome (HFRS) patients. Platelets were stimulated *ex vivo* with the agonists ADP (10 μmol/L, A), PAR-1-activating peptide (15 μmol/L, B), PAR-4-activating peptide (60 μmol/L, C) and collagen-related peptide (CRP) (0.5 μg/mL, D). The estimated marginal means were calculated using the generalized estimating equation (GEE) for percentage of activated platelets as measured by flow cytometric analysis of exposure of P-selectin on the platelet surface relative to isotype control. Error bars indicate the standard error of the mean. The number of study individuals with one or more samples included in each time point: 1-7 days post disease onset (DPDO) n = 10; 8-14 DPDO n = 5; 15-30 DPDO n = 3 and > 31 DPDO n = 12. The GEE method was used to calculate whether percentage P-selectin positive platelets differed between each time point compared to follow up (> 31 DPDO; shown as #). * P < .05; ** P < .01; *** P < .001.
Figure 6. Platelet activation in patients with disseminated intravascular coagulation and thromboembolic complications. The estimated marginal means was calculated for sP-selectin (A and C) and sGPVI (B) using the generalized estimating equation method (GEE) for HFRS patients that fulfilled disseminated intravascular coagulation (DIC) (A and B) or had radiologically verified thromboembolic complications (C). Error bars indicate the standard error of the mean. The numbers underneath each time point represents the number of study individuals with one or more samples included in that time point. The difference in these markers in patients that fulfilled DIC criteria or had thromboembolic complications (thrombosis) was compared to patients that did not fulfil DIC criteria (no DIC) or had thromboembolic complications (no thrombosis) within each given time point using the GEE method. * $P < .05$; ** $P < .01$; *** $P < .001$. 
Supplementary Methods

Soluble GPVI quantification by ELISA
sGPVI levels were measured by a newly developed ELISA. 96 well standard binding plates from MesoScale Discovery (MSD) (Rockville, MD) were coated overnight at 4°C with 4 µg/mL sheep anti-human GPVI polyclonal antibody (R&D Systems, Abingdon, UK). The plate was blocked with 5% MSD Blocker A for 1 hr at RT, washed ×3 with 150 µL PBS / 0.05% Tween (PBST) and 25 µl of undiluted platelet poor plasma added to duplicate wells. Samples were incubated at RT with vigorous shaking for 1 hr. The plate was washed ×3 with PBST. Biotinylated sheep anti-human GPVI antibody was diluted to 1 µg/mL in 1% MSD Blocker A and 25 µL added to each well. The plate was incubated at RT for 1 hr with shaking at 650rpm then washed ×3 with PBST. 150 µL 2× read buffer was added to each well and the plate read on a MesoScale Quickplex SQ120 Plate Scanner according to the manufacturer’s instructions.

Platelet ex vivo activation measured by flow cytometry
Fluorescein (FITC)-conjugated chicken antibodies towards human fibrinogen, human P-selectin (CD62P) and the negative isotype control (IgY) antibody were from Diapensia HB (Linköping, Sweden). Chemicals for the HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl2, 5.6 mmol/L glucose, 1 g/L bovine serum albumin and 20 mmol/L HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]), pH 7.40), EDTA, apyrase (grade VII) and ADP (adenosine 5’-diphosphate monopotassium salt dihydrate) were from Sigma Chemical Company (St. Louis, MO, USA). The ADP powder was dissolved in NaCl 9mg/ml to a concentration of 0.13 mmol/L. The PAR1 thrombin-receptor-activating peptide (PAR1-AP, amino acid sequence SFLLRN) and the PAR4 thrombin receptor activating peptide
(amino acid sequence AYPGKF) were purchased from JPT peptide technologies (Berlin, Germany) and were dissolved in NaCl 9 mg/mL to a concentration of 0.195 mmol/L (PAR1-AP), 5.2 or 0.78 mmol/l (PAR4-AP). A collagen-related peptide (CRP) with the sequence Gly-Cys-Hyp-(Gly-Pro-Hyp)_{10}-Gly-Cys-Hyp-Gly-NH_2 was purchased from JPT peptide technologies (Berlin, Germany). The peptide solution was diluted to 25.5 μg/mL in 0.05% HAc. All solutions were aliquoted and stored at -70°C and thawed immediately before use. The CRP peptide was further diluted 1:4 in HEPES buffer before the start of the experiment.

Platelet activation was measured as platelet-bound fibrinogen or P-selectin, utilising flow cytometry. Blood samples were drawn into vacutainers containing sodium citrate and inverted gently 10 times to ensure proper mixing of blood with anti-coagulants. The anti-coagulated blood sample was run after one hour. 5 μL of well mixed whole blood was transferred to plastic tubes containing saturating concentrations of FITC-conjugated antibody against human fibrinogen, human P-selectin or a corresponding concentration of an isotype control antibody (FITC-IgY) and HEPES buffer to a final volume of 60 μL. Apyrase grade VII (Sigma Chemical Company, St. Louis, MO, USA), final concentration 0.2 U/mL, was added to some tubes in order to estimate the ADP contribution to fibrinogen binding. After 10 minutes, 10 μL of ADP, CRP, PAR1-AP, PAR4-AP or buffer was added to the samples at exact time intervals. Final concentrations were 10 μmol/L of ADP, 15 μmol/L of PAR1-AP, 60 or 400 μmol/L of PAR4-AP (for P-selectin expression and fibrinogen binding, respectively) and 0.5 μg/mL of CRP.

After exactly 10 minutes, the reaction was stopped by the addition of 1000 μL of HEPES buffer. All steps were performed at room temperature and without stirring the samples. A discrimination frame was set around the platelet cluster using forward and side scatter characteristics. An analytical marker was set in the FL1 fluorescence channel (FITC) to divide
gated particles in samples with the IgY control antibody with corresponding fluorescence intensity into two fractions, one that contained 98.5-99.5% of the platelets and the other containing the brightest 0.5-1.5 % of the platelets. For fibrinogen binding, the marker was set using a sample with 10 mmol/L EDTA in the buffer to prevent fibrinogen from binding to the GPIIb/IIIa receptor. Platelets with fluorescence intensity higher than the marker were identified as fibrinogen binding or P-selectin expressing cells. Results are expressed as percentage activated platelets compared to the baseline sample and is based on analysis of 10 000 platelets.

The difference between percentage positive platelets in non-stimulated platelets as measured by P-selectin platelet surface exposure during disease compared to follow up was tested using Wilcoxon signed rank test for paired samples.
Supplementary Figure 1. Platelet counts for patients with hemorrhagic fever with renal syndrome. (A) Platelet counts per day following disease onset for all patients until 60 days post disease onset. Reference lines illustrating the lower (145×10⁹/L) and upper (387×10⁹/L) reference range for platelet counts is shown. (B) The minimum platelet value for each patient obtained within 10 days post disease onset. A line illustrating the level of 100×10⁹/L platelets is inserted. (C) The maximum platelet count for each patient obtained within 30 days post disease onset. The limit for thrombocytosis (400×10⁹/L) is included for orientation.
Supplementary Figure 2. Platelet regeneration and activation association with maximum DIC score in hemorrhagic fever with renal syndrome patients. The mean (± SEM) maximum level of (A) TPO, (B) IPF, (C) MPV, (D) sP-selectin and (E) sGPVI relative to the maximum DIC score for each patient. Only factors with a minimum of two samples during disease were included. The association between the specified factors and maximum DIC score is analysed for statistical significance using spearman’s rank correlation coefficient. * $P < .05$; ** $P < .01$; *** $P < .001$. 
Supplementary Table 1. Association between platelets and platelet regeneration markers in hemorrhagic fever with renal syndrome patients*

<table>
<thead>
<tr>
<th></th>
<th>Immature platelet fraction (%)</th>
<th>Mean platelet volume (fL)</th>
<th>Thrombopoietin (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (×10⁹/L)</td>
<td>β = - 0.018, P &lt; .001</td>
<td>β = - 0.005, P &lt; .001</td>
<td>β = - 0.747, P &lt; .001</td>
</tr>
<tr>
<td>Immature platelet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fraction (%)</td>
<td>β = 0.234, P &lt; .001</td>
<td>β = 0.016, P = .001</td>
<td></td>
</tr>
<tr>
<td>Mean platelet volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(fL)</td>
<td>β = 0.005, P &lt; .001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All values up to and including 16 days post disease onset are included. The estimated β-coefficients from the generalized estimating equations analysis is given along with the p-value. This corresponds to the change in levels of platelets, TPO, IPF or MPV for one unit increase for continuous covariates (slope).
Supplementary Table 2. Platelet production associates with platelet activation in hemorrhagic fever with renal syndrome patients*

<table>
<thead>
<tr>
<th></th>
<th>sP-selectin (ng/mL)</th>
<th>sGPVI (ng/mL)</th>
<th>vWF (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelets</strong> (×10^9/L)</td>
<td>β = 0.075</td>
<td>β = 0.06</td>
<td>β = -0.001</td>
</tr>
<tr>
<td></td>
<td>P &lt; .001</td>
<td>P = .005</td>
<td>P = .304</td>
</tr>
<tr>
<td><strong>Thrombopoietin</strong> (pg/mL)</td>
<td>β = -0.079</td>
<td>β = -0.008</td>
<td>β = -0.001</td>
</tr>
<tr>
<td></td>
<td>P &lt; .001</td>
<td>P &lt; .001</td>
<td>P = .983</td>
</tr>
</tbody>
</table>

* All values up to and including 16 days post disease onset are included. The estimated β-coefficients from the generalized estimating equations analysis are given along with the p-value. This corresponds to the change in levels of sP-selectin, sGPVI and vWF for one unit increase for the continuous covariates (slope): platelet count and thrombopoietin.
Supplementary Table 3. Analysis for *ex vivo* platelet activation in hemorrhagic fever with renal syndrome patients*

<table>
<thead>
<tr>
<th>P-selectin exposure</th>
<th>Platelet activation (%)</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IgY†</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td>1.55 (1.51 – 1.59)</td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td>1.5 (1.46 – 1.56)</td>
<td>-</td>
</tr>
<tr>
<td><strong>HEPES‡</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td>6.1 (3.43 – 8.28)</td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td>2.61 (2.22 – 4.73)</td>
<td>.026</td>
</tr>
<tr>
<td><strong>PAR4-activating peptide§</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td>27.56 (14.01 – 54.88)</td>
<td></td>
</tr>
<tr>
<td>Follow-up</td>
<td>84.5 (71.72 – 90.88)</td>
<td>.002</td>
</tr>
</tbody>
</table>

*Results from the first platelet function analysis is included (disease) and compared to follow up results, which are obtained at least 90 days post disease onset. The difference between disease and follow up for each stimulation is analyzed using Wilcoxon signed rank test and the corresponding P-value is shown. Values are shown as median (interquartile range).† The IgY (isotype control) samples are manually gated to fall within 1-2% activated platelets and function as reference parameters. All other parameters are shown relative to this reference parameter.‡ HEPES buffer indicates the pre-activation state of platelets for the P-selectin analysis. One patient was excluded due to technical problems.§ PAR4-activating peptide is used as a positive control in this analysis.
Supplementary Table 4. Reduced fibrinogen binding following degradation of platelet-released ADP upon stimulation*

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Percentage activated platelets</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (10 μmol/L)</td>
<td>65.1 (45 – 81.5)</td>
<td>.002</td>
</tr>
<tr>
<td>+ apyrase‡</td>
<td>4.6 (1.5 – 6.4)</td>
<td></td>
</tr>
<tr>
<td>PAR1-activating peptide (15 μmol/L)</td>
<td>51.5 (17 – 70.5)</td>
<td>.002</td>
</tr>
<tr>
<td>+ apyrase</td>
<td>27.8 (8.3 – 41)</td>
<td></td>
</tr>
<tr>
<td>PAR4-activating peptide (400 μmol/L)</td>
<td>93.3 (67.8 – 94.2)</td>
<td>.002</td>
</tr>
<tr>
<td>+ apyrase</td>
<td>71.3 (36.3 – 76.4)</td>
<td></td>
</tr>
<tr>
<td>Collagen related peptide (0.5 μg/mL)</td>
<td>36.9 (9.4 – 77.1)</td>
<td>.002</td>
</tr>
<tr>
<td>+ apyrase</td>
<td>14.9 (4.9 – 38.2)</td>
<td></td>
</tr>
</tbody>
</table>

* Ex vivo stimulation of platelets as determined by fibrinogen binding at the first time point available for 12 HFRS patients. Values are shown as median (interquartile range).
† The percentage activated platelets following stimulation with or without addition of apyrase is compared using Wilcoxon-signed rank test for paired samples.
‡ Apyrase cleaves ADP, which is responsible for enhanced platelet activation following platelet receptor stimulation. Data on ADP + apyrase is included in the table to show that apyrase was effective in degrading ADP under these conditions.