





## BRIEF REPORTS

# Anti-platelet factor 4/heparin antibodies in patients with Hantaan virus infection

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## Funding information

National Natural Science Foundation of China, Grant/Award Number: 81771705 and 81871239; Natural Science Foundation of Shaanxi Province, Grant/Award Number: 2017SF-196

**Handling Editor:** Pantep Angchaisuksiri

## Abstract

**Background:** Hemorrhagic fever with renal syndrome (HFRS) induced by Hantaan virus infection and heparin-induced thrombocytopenia (HIT) are associated with symptoms such as thrombocytopenia and thrombosis. However, related molecules, such as anti-platelet factor 4 (PF4)/heparin antibodies, in patients with HFRS have not been evaluated.

**Objectives:** To test plasma levels of anti-PF4/heparin antibodies and study the possible role of these antibodies in HFRS pathogenesis.

**Methods:** Indirect ELISA was used to determine plasma levels of anti-PF4/heparin antibodies in 75 patients with HFRS and 20 normal controls. The 4Ts (thrombocytopenia, timing of platelet count fall, thrombosis or other sequelae, and other causes of thrombocytopenia) scoring system was used to determine the probability of HIT occurrence. A PF4-enhanced platelet activation assay was used to detect the pathological effects of anti-PF4/heparin antibodies. The laboratory/clinical features and viral load of all the patients were also assessed.

**Results:** Of the 75 patients with HFRS enrolled in this study, 69 had thrombocytopenia. Platelet count was negatively correlated with Hantaan viral load. Moreover, the optical density (OD) values of plasma antibodies against PF4/heparin in normal controls were less than 0.65, 4 patients tested strongly positive for anti-PF4/heparin antibodies (OD values, 1.51–3.87), 21 patients were weakly positive (OD values, 0.66–0.74), and 50 patients were negative (OD values, 0.16–0.65). Moreover, all 4 patients who tested strongly positive for anti-PF4/heparin antibodies showed a low probability of HIT (4Ts score of 3 or less) and had negative results in the PF4-enhanced platelet activation assay.

**Conclusions:** Hantaan virus infection produces nonpathogenic antibodies against PF4/heparin; however, the generation mechanism of these antibodies requires further study.

Meng Wang and Chun-mei Zhang contributed equally to this work.

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## KEYWORDS

anti-platelet factor 4 (PF4)/heparin antibodies, Hantaan virus, hemorrhagic fever with renal syndrome, heparin-induced thrombocytopenia, thrombocytopenia, thrombosis

## Essentials

- Anti-platelet factor 4 (PF4)/heparin antibodies have not been evaluated in patients with hemorrhagic fever with renal syndrome (HFRS).
- One-third of patients with HFRS were positive for anti-PF4/heparin antibodies.
- Four patients with high optical density values for anti-PF4/heparin antibodies tested negative in functional tests.
- The generation mechanism of anti-PF4/heparin antibodies in patients with HFRS needs further study.

## 1 | INTRODUCTION

The Hantaan virus belongs to the genus *Hantavirus* of the Bunyaviridae family. It can lead to serious fatal hemorrhagic fever with renal syndrome (HFRS) in humans with clinical characteristics manifested by fever, thrombocytopenia, hemorrhage, and acute renal injury.<sup>1</sup> Approximately 25% of patients with HFRS present with coagulation system activation, fibrinolytic system disorders, fibrin deposition, and multiorgan micro-thrombus formation.<sup>2,3</sup> However, the causes of thrombocytopenia and thrombosis during HFRS have not been fully declared.

Heparin-induced thrombocytopenia (HIT) is a clinical-pathological disorder of thrombotic thrombocytopenia triggered by antibodies against platelet factor 4 (PF4)/heparin that recognize PF4 and heparin complexes.<sup>4,5</sup> After binding with PF4/heparin complexes, the fragment crystallizable (Fc) region of anti-PF4/heparin antibodies can bind to the FcγRIIIa receptor on platelets. As a result, platelets release more PF4s, which promote the formation of large immune complexes in the blood. These compounds consume more platelets, which result in thrombocytopenia. Nevertheless, some studies have found that HIT can also occur in patients without heparin exposure, which is called spontaneous HIT syndrome or auto-immune HIT.<sup>6</sup> RNA and DNA induced by bacterial/viral infection or trauma can also contribute to the generation of platelet-activating antibodies.<sup>7,8</sup> Recent studies have shown that anti-PF4/heparin antibodies developed in coronavirus disease 2019 or after ChAdOx1 nCoV-19 vaccination.<sup>9,10</sup> However, anti-PF4/heparin antibodies have not been evaluated in patients with HFRS.

Here, we tested plasma levels of antibodies against PF4/heparin and studied the possible role of these antibodies in HFRS pathogenesis.

## 2 | METHODS

### 2.1 | Enrolled subjects

In total, 142 blood samples from 75 patients with HFRS at Tangdu Hospital of the Air Force Medical University (Xi'an, China) and Xi'an Eighth Hospital in 2020 were used in this study. All patients were diagnosed with HFRS due to the presence of IgG- and IgM-specific antibodies against the Hantaan virus. Patients with other diseases

were excluded from the study. Depending on the Chinese diagnostic criteria for HFRS, the severity of the disease can be divided into mild, moderate, severe, and critical<sup>11</sup> as follows: (i) mild: mild kidney damage and no obvious oliguric phase; (ii) moderate: obvious symptoms of hemorrhage (skin and mucous membrane), effusion (bulbar conjunctiva), uremia, and kidney failure with a significant oliguric phase; (iii) severe: severe uremia, effusion, hemorrhage, and kidney failure with oliguria (urine output, 50–500 ml/day) for ≤5 days or anuria (urine output, <50 ml/day) for ≤2 days; (iv) critical: patients who had more than one of the following symptoms: pulmonary edema, brain edema, severe secondary infection, visceral hemorrhage, refractory shock, heart failure, and severe renal failure with oliguria (urine output, 50–500 ml/day) for more than 5 days, anuria (urine output, <50 ml/day) for more than 2 days, or a blood urea nitrogen level of greater than 42.84 mmol/L.

As previously described,<sup>12</sup> the typical course of HFRS is divided into five sequential stages (febrile, hypotensive, oliguric, diuretic, and convalescent), and the febrile, hypotensive, and oliguric stages are usually classified as the acute phase; the diuretic and convalescent stages are usually classified as the convalescent phase. Generally, we collected patients' blood samples in chronological order, and the time points for sample collection were 3–12 days after HFRS onset for the acute phase and more than 13 days for the convalescent phase. Additionally, 20 healthy volunteers at the Department of Immunology of Basic Medicine School at Air Force Medical University were enrolled in this study as controls. The characteristics of the enrolled participants are summarized in Table 1. This study was approved by the Institutional Review Board of the Air Force Medical University (KY20173178-1).

### 2.2 | Sample collection

The plasma samples were separated from EDTA anticoagulation peripheral blood samples by centrifugation (1000g, 15 min) and cryopreserved at −80°C before application. Platelet-rich plasma (PRP) was obtained using successive centrifugation, and acid-citrate-dextrose solution-treated peripheral blood samples from healthy volunteers who did not take nonsteroidal anti-inflammatory drugs or antiplatelet medications in the past 10 days were centrifuged at

**TABLE 1** Characteristics of enrolled subjects

Characteristics	Number of patients	Number of controls
Disease severity		
Mild	10	-
Moderate	34	-
Severe	21	-
Critical	10	-
Phase of disease		
Acute	105	-
Convalescent	37	-
Age		
Range	9–78	20–55
Median	41	37
Sex		
Male	59	10
Female	16	10
Thrombocytopenia	69	0
Renal failure	34	0
Shock	11	0
Hemorrhage	44	0
Thrombus	4	0
Cardiac insufficiency	11	0
Pulmonary edema	4	0

Note: The HFRS disease severity was divided into mild, moderate, severe, and critical: (i) mild: mild kidney damage and no obvious oliguric phase; (ii) moderate: obvious symptoms of hemorrhage (skin and mucous membrane), effusion (bulbar conjunctiva), uremia, and kidney failure with a significant oliguric phase; (iii) severe: severe uremia, effusion, hemorrhage, and kidney failure with oliguria (urine output, 50–500 ml/day) for  $\leq 5$  days or anuria (urine output,  $< 50$  ml/day) for  $\leq 2$  days; (iv) critical: patients who had more than one of the following symptoms: pulmonary edema, brain edema, severe secondary infection, visceral hemorrhage, refractory shock, heart failure, and severe renal failure with either oliguria (urine output, 50–500 ml/day) for  $> 5$  days, anuria (urine output,  $< 50$  ml/day) for  $> 2$  days, or a blood urea nitrogen level of  $> 42.84$  mmol/L. Thrombocytopenia: platelet count  $< 100 \times 10^9$ /L. All patients and controls were Chinese of Han ethnicity.

160g for 10min. Platelets were isolated from PRP, washed with magnesium-free and calcium-free Tyrode's buffer containing glucose and apyrase, and resuspended in calcium- and magnesium-containing Tyrode's buffer with glucose and bovine serum albumin.<sup>13</sup>

## 2.3 | ELISA

The levels of antibodies against PF4/heparin complexes in plasma were quantified using indirect ELISA kits (KL-HIT-Hu; KALANG) following the manufacturer's protocol. Absorbance was measured at 450nm using a SpectraMax absorbance reader (Molecular Devices).

## 2.4 | PF4-enhanced platelet activation assay

PF4-enhanced platelet activation assay was performed as described previously with some modifications.<sup>14</sup> First, the plasma thawed at 37°C for 5 min and then heated (56°C, 45 min) to inactivate the remaining thrombin; then, the patient's plasma and washed platelets were incubated with either saline solution, 10  $\mu$ g/ml PF4, 0.2 U/ml low molecular weight heparin (enoxaparin), or 100IU/ml heparin on a four-channel platelet aggregation analyzer (TECHLINK BIOMEDICAL LBY-NJ4). Platelet aggregation was measured by monitoring light transmission within 10 min. The patient's plasma caused platelet aggregation in at least two donors in the presence of saline solution or PF4, which was interpreted as a positive result.

## 2.5 | The viral load detection

Plasma viral load in patients with HFRS was determined using previously established methods.<sup>15</sup>

## 2.6 | Statistical Analysis

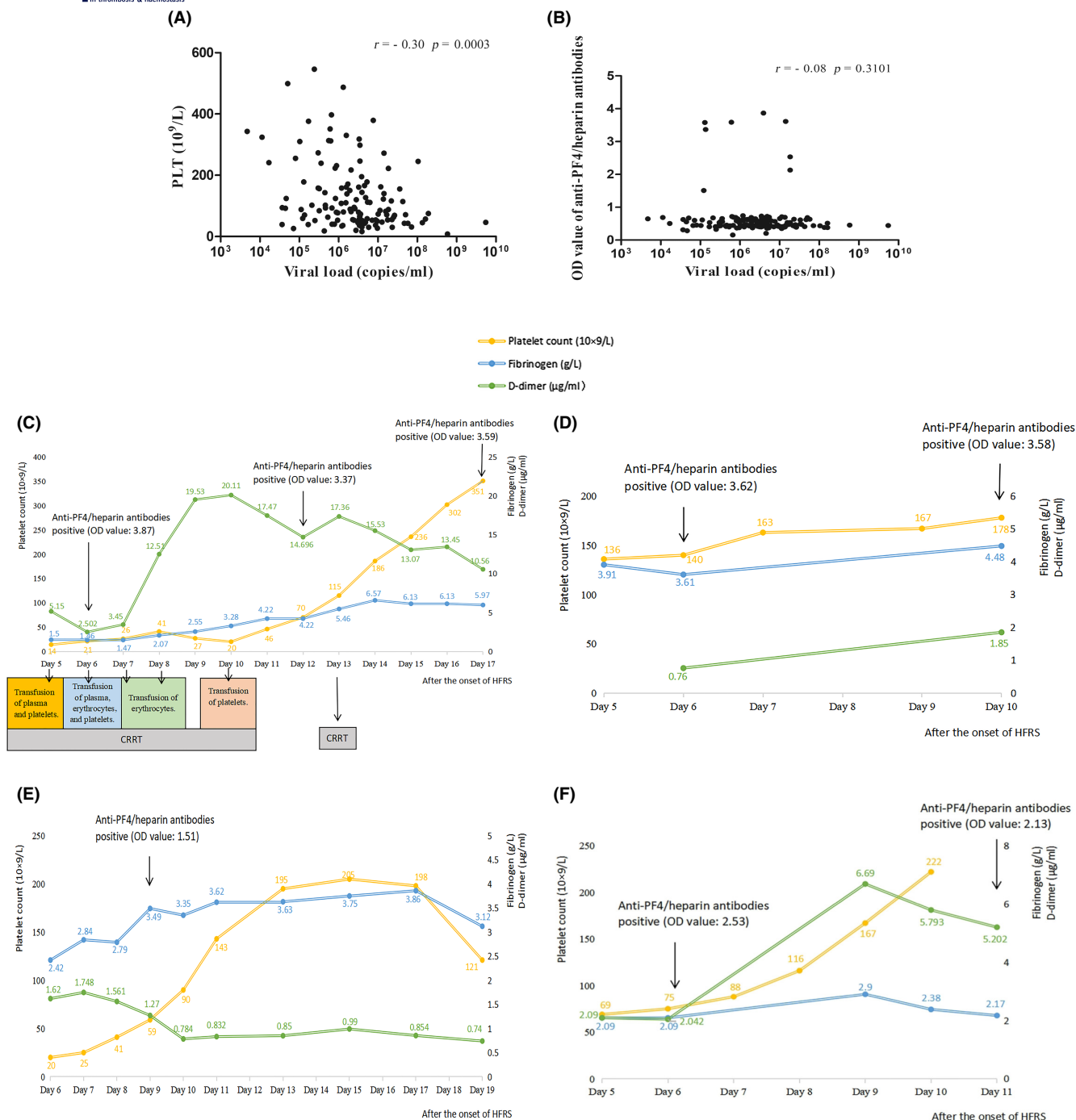
Prism version 8 (GraphPad Software) was used for statistical analysis. The Spearman correlation was used to analyze the correlation between viral load and clinical parameters. *p* value less than 0.05 was considered statistically significant.

# 3 | RESULTS AND DISCUSSIONS

First, we analyzed the laboratory characteristics of the patients with HFRS; 69 of the 75 patients in this study had the symptoms of thrombocytopenia, with platelet count less than  $100 \times 10^9$ /L. We then analyzed the correlation between platelet count and Hantaan viral load. The results showed that platelet count was negatively correlated with viral load ( $r = -0.30$ ,  $p = 0.0003$ , Figure 1A).

Next, plasma levels of anti-PF4/heparin antibodies were measured in patients with HFRS and healthy participants. The optical density (OD) values of plasma antibodies against PF4/heparin in normal controls were less than 0.65. Among the 75 patients with HFRS, four patients had strikingly high OD values of anti-PF4/heparin antibodies ranging from 1.51 to 3.87; 21 patients were weakly positive, with OD values ranging from 0.66 to 0.74; 50 patients were negative, with OD values ranging from 0.16 to 0.65; and no patients tested positive with OD values between 0.75 and 1.50 (Table 2). However, there was no statistically significant correlation between plasma levels of anti-PF4/heparin antibodies and viral load ( $r = -0.08$ ,  $p = 0.31$ , Figure 1B).

We then analyzed the symptoms of thrombocytopenia and thrombosis in four patients with strongly positive anti-PF4/heparin antibodies (Table 2). Except for Patient 2, the other three patients presented with mild-to-severe thrombocytopenia. Patients 1 and



**FIGURE 1** Laboratory and clinical parameters of patients with HFRS. Correlation between viral load and (A) platelet count and (B) OD values of anti-PF4/heparin antibodies in all patients with HFRS. The timeline of (C) Patient 1, (D) Patient 2, (E) Patient 3, and (F) Patient 4 with high OD values for anti-PF4/heparin antibodies. Laboratory features were as follows: platelet count, D-dimer level, and fibrinogen level. Time points for the results of the anti-PF4/heparin antibodies and key clinical events were also shown, including CRRT and blood component transfusion. The correlation was evaluated using the Spearman's correlation test, where  $r$  indicated the Spearman's correlation coefficient. Statistical significance was set at  $p < 0.05$ . CRRT, continuous renal replacement therapy; HFRS, hemorrhagic fever with renal syndrome; OD, optical density; PF4, platelet factor 4; PLT, platelets.

3 had severe thrombocytopenia (platelet count,  $20 \times 10^9/L$  or less), while Patient 4 had mild thrombocytopenia (platelet count,  $60\text{--}100 \times 10^9/L$ ). Moreover, all these four patients had a certain degree of abnormal coagulation, presenting as one or more abnormalities in

laboratory indicators related to thrombosis, such as thrombin time, activated partial thromboplastin time (aPTT), prothrombin time (PT), D-dimer levels, and fibrinogen concentration. These findings suggest significant coagulation activation in these four patients.

**TABLE 2** Clinical and laboratory characteristics of four patients with HFRS who tested strongly positive for anti-PF4/heparin antibodies

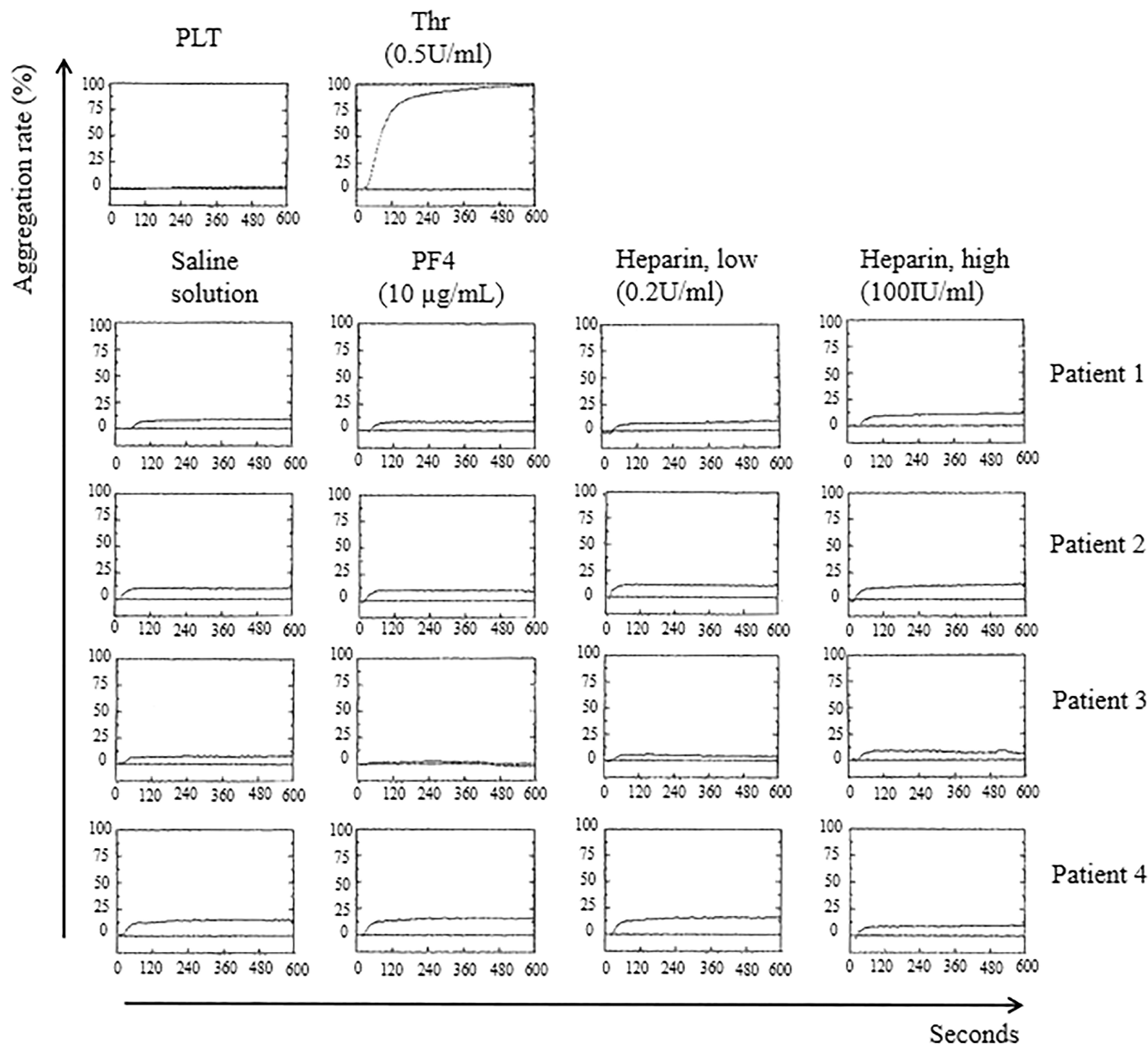
Test items	Patient 1	Patient 2	Patient 3	Patient 4
Age, years	29	73	59	50
Sex	Male	Male	Female	Male
Disease severity	Critical	Mild	Severe	Mild
Symptoms	Fever, fatigue, headache, emesis, and decreased urine output along with loose stools	Fever, fatigue, and headache	Fever, fatigue, decreased urine output, and backache	Fever, chills, headache, and muscular soreness
WBC peak ( $\times 10^9/L$ )	53.65	7.96	10.48	17.86
Platelet count nadir ( $\times 10^9/L$ )	14	136	20	69
Fibrinogen nadir (g/L)	1.47	3.48	2.42	2.09
FDP peak ( $\mu g/ml$ )	43.8	/	6.4	12.84
D-dimer peak ( $\mu g/ml$ )	20.11	1.85	1.748	6.69
INR peak	1.39	1.03	0.99	1
PT peak (s)	14.7	13.6	11	11.4
aPTT peak (s)	52.9	36.8	34.4	24.1
TT peak (s)	49.3	15.5	31.2	18.8
4Ts	1	2	2	2
Treatments	Transfusion; CRRT; ceftriaxone sodium; piperacillin-tazobactam	Ceftriaxone sodium	Cefotiam	Cefotiam; biapenem
OD values of anti-PF4/heparin antibodies	Acute: 3.87, 3.37; convalescence: 3.59	Acute: 3.62; convalescence: 3.58	Acute: 1.51; convalescence: —	Acute: 2.53; convalescence: 2.13
Detailed reports	The patient received CRRT and blood component transfusion after the onset of HFRS. During the following days, the patient's platelet count gradually increased and renal function gradually improved. He was discharged on Day 23 after HFRS onset.	Laboratory results on admission showed slightly elevated fibrinogen, as well as prolonged PT and aPTT. However, the patient's platelet count was normal throughout the hospital stay. He was hospitalized for a total of 7 days until he recovered from the illness.	Although the patient was a severe case, she had not yet met the criteria for CRRT. After prophylactic treatment of bleeding, the patient's platelet count increased and the coagulation indicator returned to normal on Day 10 after HFRS onset. She was discharged on Day 19 after the onset of HFRS.	Although the levels of patient's FDP and D-dimer were slightly higher, imageological examination ruled out any risk of thrombosis. On Day 14, laboratory examinations were almost normal except blood pressure. Finally, the patient was discharged on Day 17 after HFRS onset.

Note: Plasma levels of anti-PF4/heparin antibodies were measured by ELISA. Abbreviations: aPTT, activated partial thromboplastin time; CRRT, continuous renal replacement therapy; FDP, fibrinogen degradation product; HFRS, hemorrhagic fever with renal syndrome; INR, international normalized ratio; OD, optical density; PT, prothrombin time; TT, thrombin time; WBC, white blood cell.

Computed tomography did not show thrombus formation at any site, but they all had some degree of hemorrhage, such as petechiae or ecchymoses on the skin and mucous membrane.

We wondered whether these patients, who were positive for anti-PF4/heparin antibodies, were exposed to heparin. The administration records showed that only seven patients (Patient 1 and another six patients who were weakly positive for antibodies against PF4/heparin) received continuous renal replacement therapy (CRRT) and/or component blood transfusion several times after symptom onset, but the OD values of anti-PF4/heparin antibodies remained stable, which suggests that the production of these antibodies probably has no relationship with heparin exposure during CRRT or component

blood transfusion. Detailed reports and the timeline of four patients who tested strongly positive for anti-PF4/heparin antibodies are shown in Table 2 and Figure 1C–F. Among these four patients, the lower the platelet count, the longer the platelet recovery time and the acute-stage duration. However, there are no differences in the duration of the acute phase and the time of platelet recovery between these four patients and the rest. Moreover, we used the 4Ts (thrombocytopenia, timing of platelet count fall, thrombosis or other sequelae, and other causes of thrombocytopenia) scoring system to determine HIT probability in four patients with high OD values in anti-PF4/heparin antibody ELISA.<sup>16</sup> The results showed a low probability of HIT in four patients (4Ts score of 3 or less) (Table 2).



**FIGURE 2** The ability of plasma to aggregate platelets in the PF4-enhanced platelet activation assay. Aggregation of donor platelets after incubating with plasma from four patients with HFRS who tested strongly positive for anti-PF4/heparin antibodies was measured with a four-channel platelet aggregation analyzer, under the conditions that in the presence of saline solution, 10 µg/ml PF4, low heparin (0.2 U/ml) and high heparin (100IU/ml) concentrations. As a positive control, we added thrombin (Thr) (0.5 U/ml) to the platelet concentrates, which showed a strong aggregation reaction. Platelet suspensions alone were used as negative controls and showed no signs of aggregation. The x axis represented time, whereas the y axis represented the percentage of aggregation. HFRS, hemorrhagic fever with renal syndrome; PF4, platelet factor 4; PLT, platelets.

Although both HFRS and HIT have thrombocytopenia and thrombosis symptoms, they differ in that hemorrhage predominates in HFRS, whereas thrombosis predominates in HIT and differ in time of onset, duration, severity, and therapy. For onset and duration, typical HIT occurs 5–10 days after heparin exposure, with more than 50% platelet count reduction. In autoimmune HIT (aHIT), the onset of thrombocytopenia varies by type, ranging from weeks to months. In HFRS, thrombocytopenia occurs in the early stages of Hantaan virus infection, and hemorrhagic diseases often occur in the hypotensive stage. For severity, patients with HIT usually have symptoms

of moderate thrombocytopenia (platelet count,  $20\text{--}60 \times 10^9/\text{L}$ ) and typically do not have bleeding complications. In aHIT, serious thrombocytopenia (platelet count, less than  $20 \times 10^9/\text{L}$ ) and vascular thrombosis can occur even without heparin exposure. Studies have shown that in HFRS, thrombocytopenia accompanied by bleeding often occurs during the acute stage with reduced platelet count, even less than  $20 \times 10^9/\text{L}$ . For therapy, studies have shown that rapid recovery of platelet count in HIT can be blocked by stopping the use of heparin or high-dose intravenous immunoglobulin. However, aHIT can last for several weeks or worsen despite heparin



discontinuation. In HFRS, platelet count will recover spontaneously in the convalescent phase or after platelet transfusion in critical cases.<sup>17-19</sup> These differences further suggest that anti-PF4/heparin antibodies in HFRS and HIT may have different functions and generation mechanisms.

We then performed a PF4-enhanced platelet activation assay to confirm whether these antibodies from the four strongly positive patients could activate platelets. The results showed that all four patients with HFRS with high OD values for anti-PF4/heparin antibodies tested negative in the PF4-enhanced platelet activation assay (Figure 2). These results suggest that the presence of anti-PF4/heparin antibodies in plasma of patients with HFRS may be nonpathogenic.

The generation of anti-PF4/polyanion antibodies is an ancient host defense mechanism.<sup>20</sup> It has been reported that the positivity rate of anti-PF4/heparin antibodies in blood bank donors is 4.3% (95% confidence interval, 3.7%–5.0%). Nevertheless, the incidence of strongly positive anti-PF4/heparin antibodies in blood bank donors is only 0.3% (OD, 1.00 or greater), which is extremely uncommon in healthy populations.<sup>21</sup> In the present study, 4 of 75 patients (5.3%) were strongly positive for anti-PF4/heparin antibodies (OD greater than 1.00, the highest value being 3.87), which was nearly 17 times than those in normal individuals. These results suggest that the high frequency of anti-PF4/heparin antibodies may be due to the Hantaan virus infection.

Besides heparin, some heparin-independent polyanions, such as hypersulfated chondroitin sulfate, pentosan polysulfate, RNA, and DNA, can also cause the generation of platelet-activating antibodies.<sup>22-24</sup> Moreover, viral or bacterial infections are primary triggers for the HIT-like immune response. It is reported that viruses and nucleic acids generated during viral infection may be the source of polyanions.<sup>20</sup> In our previous study, plasma levels of cell-free DNA (cf-DNA) in patients with HFRS, which peaked at the hypotensive phase and declined during the convalescent phase, correlated positively with viral load, indicating a promising DNA damage response triggered by viral infection.<sup>25</sup> These results suggest that Hantaan virus RNA and cf-DNA in the acute stage might be one of the possible factors inducing anti-PF4/heparin antibodies in HFRS. However, we found no correlation between viral load and anti-PF4/heparin antibodies levels in our study, which implies that the generation mechanism of anti-PF4/heparin antibodies in HFRS needs to be explored in the future.

## 4 | CONCLUSION

Hantaan virus infection results in the production of nonpathogenic antibodies against PF4/heparin, but the generation mechanism of these antibodies requires further study.

## AUTHOR CONTRIBUTIONS

Meng Wang and Chun-mei Zhang performed the experiments and analyzed the data. Ying Ma, Kang Tang, Xi-yue Zhang, Ran Zhuang,

and Bo-quan Jin contributed to reagents/analysis tools. Xiao-zhou Jia and Hai-feng Hu contributed to sample collection. Meng Wang and Yun Zhang wrote the paper. Ran Zhuang, Bo-quan Jin, and Yun Zhang revised the paper; Yu-si Zhang and Yun Zhang conceived and designed the tests. The manuscript was read and approved by all authors.

## FUNDING INFORMATION

This study was funded by the National Natural Science Foundation of China (No. 81771705 and No. 81871239) and the Natural Science Foundation Research Project of Shaanxi Province in China (No. 2017SF-196).

## RELATIONSHIP DISCLOSURE

All authors declare no possible conflicts of interest.

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**How to cite this article:** Wang M, Zhang C-m, Ma Y, et al. Anti-platelet factor 4/heparin antibodies in patients with Hantaan virus infection. *Res Pract Thromb Haemost.* 2022;6:e12813. doi: [10.1002/rth2.12813](https://doi.org/10.1002/rth2.12813)