Cerebral venous sinus thrombosis and thrombocytopenia due to heparin-independent anti-PF4 antibodies after adenovirus infection

Cerebral venous sinus thrombosis (CVST) is a rare cerebrovascular disorder predominantly observed in adults, but can pose unique challenges in pediatric patients.[1] The underlying etiologies of CVST are diverse, encompassing hypercoagulable states, infections, and trauma. While concomitant thrombocytopenia is uncommon in CVST cases,[2] instances of CVST and thrombocytopenia have been reported following administration of adenoviral vector-based vaccines, ChAdOx1 nCoV-19 and Ad26.COV2.S.[3-5] This condition has been termed vaccine-induced immune thrombotic thrombocytopenia (VITT), which is caused by antibodies against platelet factor 4 (PF4). In this paper, we report on a pediatric case of severe CVST and thrombocytopenia, emerging 1 week after an adenovirus infection. Recurrent occlusion and profound thrombocytopenia, which responded to intravenous immunoglobulin (IVIG) therapy, was observed. Platelet-activating anti-PF4/heparin antibodies were identified in patient's serum. Informed signed consent was obtained from the parents of the patient to publish the case.

Our patient is a 7-year-old girl who presented to the family physician with a sudden onset of severe headache and vomiting. One week prior, she had fever and conjunctivitis. There was no cranial trauma in the medical history. She was initially hospitalized in a regional hospital where computed tomography (CT) detected bilateral frontal subdural hematoma. Laboratory investigation showed isolated thrombocytopenia ($11x10^9$/L). After 2 days of hospitalization, she was transferred to the pediatric intensive care unit at our university hospital. At admission, D-dimer was 54 μg/mL fibrinogen equivalent units (FEU) and fibrinogen (40 mg/dL) as well as factor XIII (33%) were decreased. Infection-associated immune thrombocytopenia was suspected. Anti-platelet antibody presence, which was tested using the monoclonal antibody immobilization of platelet antigens assay, and anti-phospholipid antibodies were not detected. Adenovirus infection was detected in the throat swab by polymerase chain reaction (PCR). PCR for following viruses were negative: SARS-CoV-2, Influenza A, Influenza B, respiratory syncytial virus, human papillomavirus, rhinovirus, herpes simplex virus-1 (HSV-1), HSV-2, enterovirus, cytomegalovirus, and Epstein-Barr virus. Bacterial and fungal cultures were also negative.

Magnetic resonance imaging with angiography revealed an extensive thrombosis in the superior sagittal sinus, partially affecting the sigmoid sinus and cortical veins. On day 4, interventional thrombectomy was successfully performed, restoring orthograde drainage from the anterior through the middle and distal segments (Figure 1A, B). After the procedure, the patient experienced retroperitoneal bleeding and subsequently hemorrhagic shock, and received platelet and erythrocyte concentrates. Interventional coiling of the inferior epigastric artery was required to stop the bleeding. After thrombectomy, D-dimer reduced to 3.3 μg/mL. Cranial CT was conducted due to the deterioration of neurological symptoms, revealing re-thrombosis of a similar extent to the initial presentation, along with congestive bleeding. Thrombocytopenia, elevated D-Dimer and decreased fibrinogen levels indicated uncontrolled hypercoagulation. Therapeutic anticoagulation with unfractionated heparin (UFH) was started, and a second mechanical thrombectomy was performed on day 5. Platelet count remained below $50x10^9$/L despite several platelet transfusions. Given the challenging course with bleeding complications and the suspicion of immune thrombocytopenia, the patient received high-dose intravenous immunoglobulin (IVIG) (a total of 45g IVIG) on days 9 and 10, leading to a swift normalization of the platelet count (>$100x10^9$/L) in the following days (Figure 1C).

On day 12, hematoma evacuation and decompressive craniectomy were performed due to progressive subdural hematoma. In the following course, the patient showed gradual improvement. Sonographic monitoring showed stable retroperitoneal hematoma. Coagulation parameters upon IVIG and under anticoagulation showed significant improvement. Discharged on day 33, the patient demonstrated a cooperative demeanor, mild word-finding difficulties, rest tremor, and normal muscle tone and strength. However, concentration and perseverance were still clearly reduced. Anticoagulation was switched to low molecular weight heparin at discharge.

The rare co-occurrence of CVST with thrombocytopenia has prompted our consideration of a rare variant of heparin-induced thrombocytopenia (HIT), known as spontaneous HIT, which can manifest without prior heparin exposure. Additionally, heightened awareness in our laboratory driven by the emergence of VITT cases 2 years ago, which also often present with thrombocytopenia and CVST, led us to consider an anti-PF4 antibody related disorder. The retrospective investigation of the sample from day 5 of hospitalization showed a strong immunoglobulin (Ig)G PF4/heparin enzyme immunosorbent assay reaction (Zymutest HIA IgG, Hyphen Biomed, Neuville-sur-Oise, France, optical density (OD) 2.8; normal range, 0-0.5). A modified heparin-induced platelet activation (HIPA) assay was performed with addition of exogenous PF4 as previously described.[6] The HIPA assay was negative with low concentration of heparin making HIT very unlikely (Figure 2A). In contrast, the modified HIPA was

positive (platelet aggregation within 5 minutes [min] in the presence of PF4 and within 15-35 min without exogenous PF4), which is a serological pattern that mimics VITT (Figure 2A).[3] Antibody-induced procoagulant platelets, determined by expression of P-selectin and phosphatidylserine (PS) externalization on the platelet surface, were analyzed using flow cytometer as previously described.[3] Antibody-mediated procoagulant platelet formation was observed in the absence of heparin and was decreased in the presence of low concentration of heparin but completely inhibited by high concentration of heparin (Figure 2B). Additionally, serum-induced procoagulant phenotype was inhibited with IV.3 (Fcγ receptor IIa blocking monoclonal antibody anti-CD32 [moAb IV.3; Stemcell™ technologies, Vancouver, Canada], 20 μg/mL) or IVIG (30 μg/mL), indicating FcγRIIA dependency (Figure 2B). Increased PS externalization can

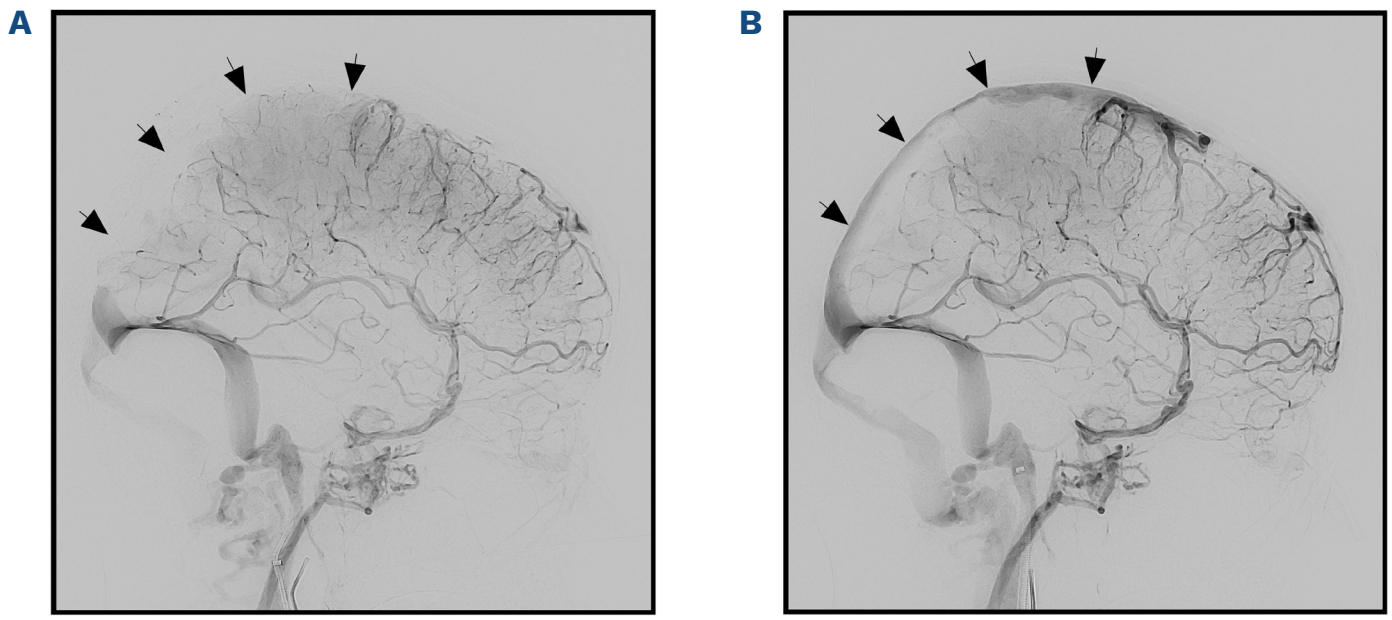

Figure 1. Radiological images and course of the platelet counts throughout the hospitalization. (A) Digital subtraction angiography was performed showing thrombosis of the superior sagittal sinus (black arrows). (B) Angiography after mechanical recanalization shows the recanalized cerebral superior sagittal sinus (black arrows). (C) Platelet count and other laboratory findings throughout hospital admission labeled with relevant interventions. Abbreviations and normal range of laboratory parameters: platelet count (normal range: 150-450x10³/μL), D-dimer (normal range: 0-0.5 μg/mL fibrinogen equivalent units), fibrinogen (normal range: 170-410 mg/dL), activated partial thromboplastin time (aPTT; normal range: <40 seconds), factor XIII (normal range: 70-140%). IVIG: intravenous immunoglobulin; EIA: enzyme immunosorbent assay; Ig: immunglobulin; HIPA: heparin-induced platelet activation; PF4: platelet factor 4.

initiate plasmatic coagulation and subsequent increased thrombin generation on the platelet surface.[8] The ability of patient's serum to induce thrombosis was investigated using a novel *ex vivo* model for antibody-mediated thrombosis (utilizing the BioFlux 200 system from Fluxion Biosciences, Alameda, USA).[7] Patient's serum induced significant thrombus formation with increased fibrin deposition compared to healthy control (mean cumulative area of thrombus [%SAC] ± standard error of mean (SEM): 2.3±0.36 *vs*. 0.9±0.2; *P*<0.01; Figure 3). Thrombus formation was markedly inhibited by IV.3 (%SAC±SEM: 0.02±0.02) and IVIG (%SAC±SEM: 1.29±0.21). Our data suggest that anti-PF4 antibodies can be generated after adenovirus infection even without a previous aberrant exposure to heparin or COVID-19 vaccine. These antibodies seem to harbor the ability of inducing thrombosis in a mechanistically similar way as VITT antibodies. Most importantly, our *ex vivo* data emphasizes the advantage of IVIG to prevent antibody-mediated thrombosis and thrombocytopenia.

Antibodies against PF4 lead to HIT and VITT, in which the immune tolerance to PF4 is disrupted, resulting in clonal expansion of B cells and subsequent secretion of anti-PF4 antibodies.[8] Recently, platelet-activating anti-PF4 antibodies were detected in an unvaccinated patient with monoclonal gammopathy and multiple thrombotic complications.[9] Similar to our case, Warkentin *et al*. published very recently two cases (a pediatric and an adult patient) developing anti-PF4 antibodies almost 1 week after adenovirus infection.[10] Both patients had thrombocytopenia and thrombotic events (fatal CVST in the child and multiple arterial and venous thrombosis in the adult). Our patient did not have previous heparin or COVID-19 vaccine exposure. HIT could also be ruled out. No additional risk factors for CVST were identified. The trigger

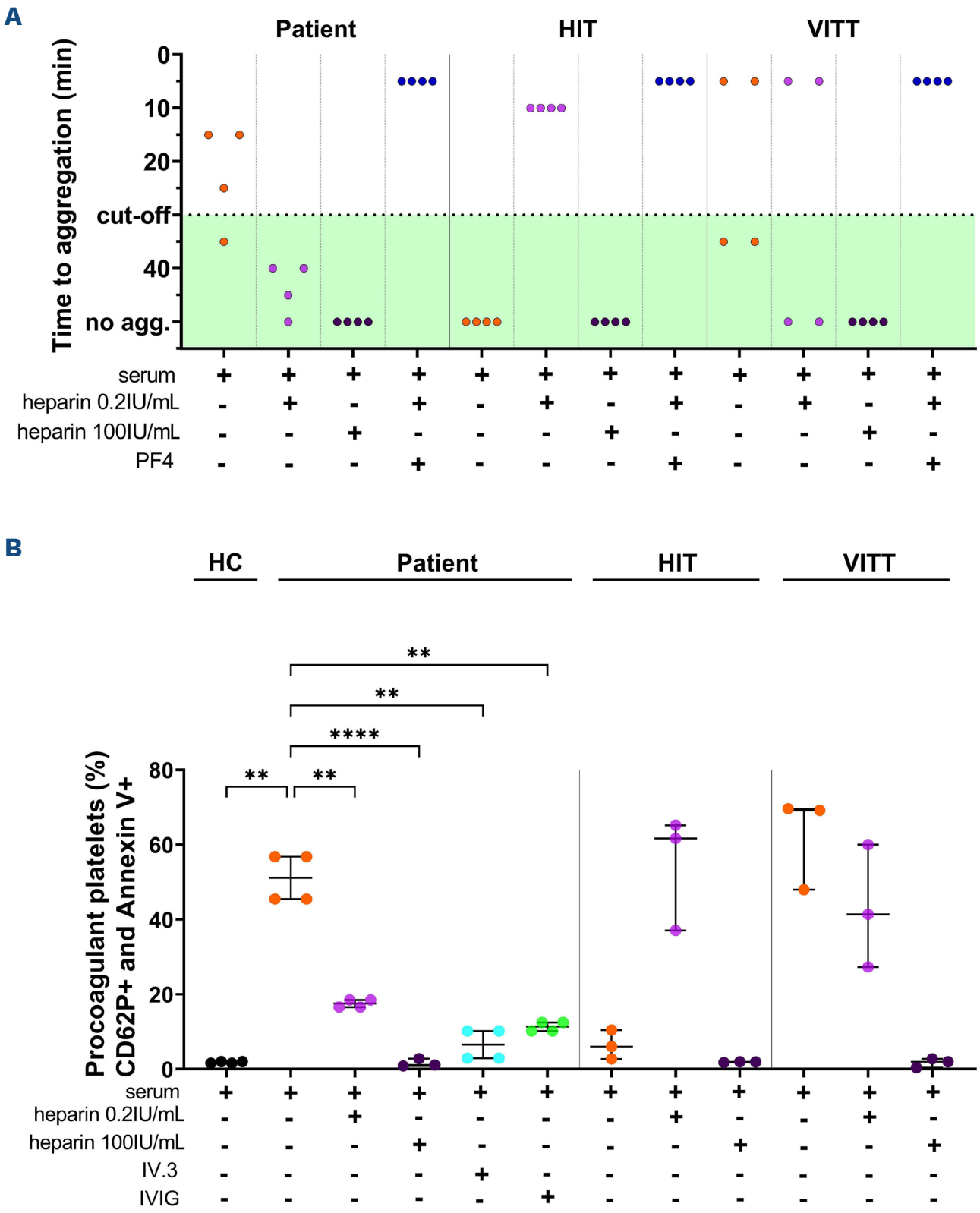

Figure 2. Patient sera induces platelet activation and procoagulant platelet phenotype. (A) Heparin-induced platelet activation (HIPA): patient serum activated platelets with exogenous platelet factor 4 (PF4) in HIPA assay, however, patient's sera did not activate platelets in the presence of low (0.2 IU/mL) or high concentration (100 IU/mL) of heparin. Historical representative samples from a heparin-induced thrombocytopenia (HIT) and a vaccine-induced immune thrombotic thrombocytopenia (VITT), patient are also depicted in the figure. (B) Flow cytometer: procoagulant platelet phenotype, determined by co-expression of P-selectin and phosphatidylserine (PS) on platelet surface, was analyzed after incubation with patient's sera. Where indicated, platelets were pretreated with IV.3 (Fcγ receptor IIA blocking monoclonal antibody) or immunoglobulin (IVIG). Patient's serum was tested with washed platelets from 4 healthy donors. Historical samples of patients with HIT (N=3) and VITT (N=3) was also shown on the figure. HC: healthy control; **P<0.01, ****P<0.0001; IVIG: intravenous immunoglobulin.

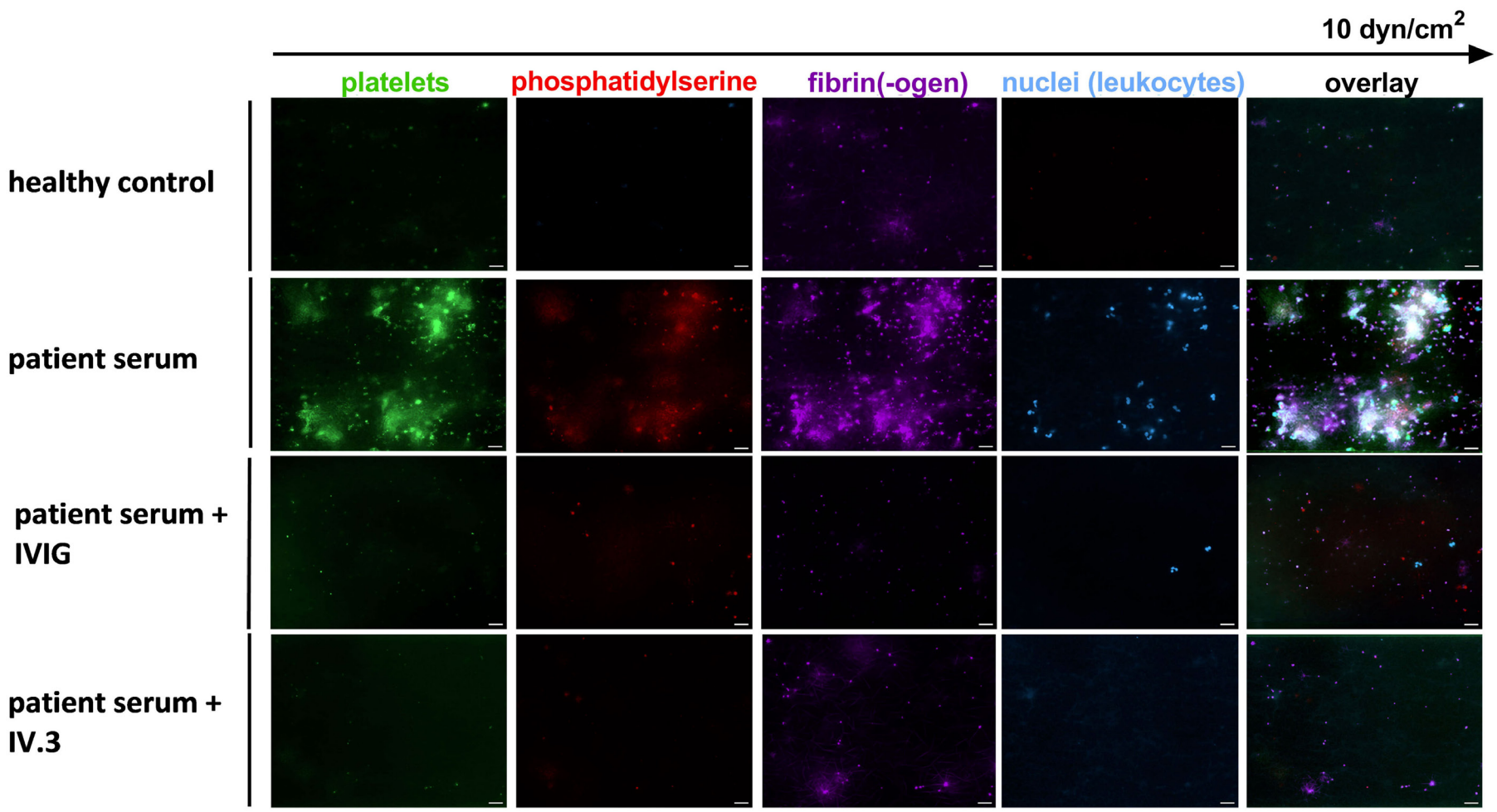

Figure 3. Patient sera induces thrombus formation *in vitro*. Platelet-rich plasma (PRP) obtained from healthy individuals in a volume of 37.5 μL was subjected to a 60-minute incubation with serum (5 μL) from the patient, all while under rotation. Following this incubation period, the samples were labeled using 3,3'-dihexyloxacarbocyaniniodid (DiOC6, 2.5 μM; Sigma Aldrich, Saint Louis, USA), Alexa Fluor (AF) 647-Annexin A5 (at a 1:200 dilution), AF 546-labeled human fibrinogen (at a concentration of 8.5 μg/mL), and Hoechst 33342 (at a concentration of 3 μg/mL; Thermo Scientific, Carlsbad, USA). When specified, the PRP was preincubated with IV.3 (20 μg/mL) or intravenous immunoglobulin (IVIG) (30 μg/mL). After the labeling procedure, the samples were reconstituted into autologous whole blood. Subsequently, the samples were recalcified and subjected to perfusion through microfluidic channels (BioFlux 200, Fluxion Biosciences, Alameda, USA) at a venous shear rate set at 250 s^{-1} (equivalent to 10 dyne/cm^2) for a duration of 10 minutes. Images were acquired at x40 magnification in different fluorescence channels using a Zeiss Axio Observer 7 microscope. The acquired images were uniformly processed using adjusted threshold settings and the exclusion of any image artefacts using of Fiji image processing software. Representative fluorescence images are shown (N=3). Scale bar 20μm.

for the development of anti-PF4/heparin antibodies in our patient is not clear. It was suggested that adenovirus or components of vaccine might be responsible for the development of anti-PF4 antibodies in VITT patients.[11] Anti-PF4 antibodies from VITT patients recognizes complexes of adenovirus hexon proteins and PF4. Furthermore, ChAdOx1 can bind to PF4 as well as coxsackievirus and adenovirus receptor (CAR), which also supports the procoagulant situation of the platelets in the case of an adenovirus infection analogous to VITT.[12] Concurrent pro-inflammatory factors may be the link to an enhanced immune response to PF4 and thus the formation of anti-PF4 antibodies in adenovirus infection.

Anticoagulation in CVST is challenging due to bleeding risk, with 40% presenting with hemorrhagic infarct at diagnosis, making immediate heparinization difficult. In our patient, the presence of intracranial hemorrhage and severe thrombocytopenia led to a cautious approach and delayed initiation of heparin. Due to high procoagulant state, patients with HIT and VITT require therapeutic anticoagulation. Heparin is, however, contraindicated in HIT. Nonetheless, successful heparin use has been reported in VITT[13] and therapeutic dose heparin can disrupt the interaction between VITT antibodies and PF4 *in vitro*.[14] Importantly, our patient, despite high anti-PF4/heparin antibodies, didn't develop new thrombosis after heparin treatment and platelet count remained stable. IVIG is a well-established first-line treatment for patients with ITP, but more recently it has been of increasing interest in the treatment of HIT and VITT.[15] IVIG mitigates platelet activation via competitive FcγRIIA binding.[16] In VITT patients with CVST, IVIG therapy correlated with lower mortality.[13] We observed a rapid increase of platelet count after IVIG therapy (Figure 1C), supporting an immune-mediated platelet activation as the underlying mechanism of the thrombocytopenia. IVIG can be considered in patients with antibody-mediated platelet activation and thrombocytopenia.

Our current study confirms the recent findings[10] that anti-PF4 antibodies may be responsible for a severe thromboembolic complication such as CVST and thrombocytopenia after adenovirus infection in the absence of prior exposure to heparin or COVID-19 vaccine. This case underscores the importance of studying the role of anti-PF4 antibodies in thrombotic events beyond HIT and VITT. Further research

is required to elucidate the underlying mechanisms, which could potentially impact the management of patients with unexplained thrombosis and thrombocytopenia.

Authors

Günalp Uzun,[1,2] Jan Zlamal,[1,2] Karina Althaus,[1,2] Andrea Bevot,[3] Florian Hennersdorf,[4] Nina Wolska,[1] Anna Jock,[5] Jan Kern,[5] Vanya Icheva,[5] Sven Poli,[6,7] Ulrike Ernemann,[4] Andreas Neu[8] and Tamam Bakchoul[1,2]

[1]Institute for Clinical and Experimental Transfusion Medicine, University Hospital of Tübingen; [2]Center for Clinical Transfusion Medicine; [3]Department of Neuropediatrics and Developmental Medicine, University Children's Hospital, University of Tübingen; [4]Department of Diagnostic and Interventional Neuroradiology, University Hospital Tübingen; [5]Department of Pediatric Cardiology, Pulmonology and Intensive Care Medicine, University Children's Hospital, University of Tübingen; [6]Department of Neurology & Stroke, University Hospital of Tübingen; [7]Hertie Institute for Clinical Brain Research, Eberhard-Karls University and [8]Department of Pediatric Endocrinology and Diabetes, University Children's Hospital, University of Tübingen, Tübingen, Germany

Correspondence:
T. BAKCHOUL - tamam.bakchoul@med.uni-tuebingen.de

https://doi.org/10.3324/haematol.2023.284127

Received: August 21, 2023.
Accepted: October 19, 2023.
Early view: October 26, 2023.

Disclosures

TB has received research funding from CoaChrom Diagnostica GmbH, DFG, Robert Bosch GmbH, Stiftung Transfusionsmedizin und Immunhämatologie e.V., Ergomed, Surrey, DRK Blutspendedienst, Deutsche Herzstiftung, Ministerium für Wissenschaft, Forschung und Kunst Baden-Württemberg; has received lecture honoraria from Aspen Germany GmbH, Bayer Vital GmbH, CSL Behring, Bristol-Myers Squibb GmbH & Co., Doctrina Med AG, Meet The Experts Academy UG, StreamedUp, Stago GmbH, Mitsubishi Tanabe Pharma GmbH and Novo Nordisk Pharma GmbH; has provided consulting services to Terumo; has provided expert witness testimony relating to heparin-induced thrombocytopenia (HIT) and non-HIT thrombocytopenic and coagulopathic disorders; has a pending patent for a flow cytometry-based assay to detect HIT and VITT (all of these are outside the current work). SP is a steering committee member of the International Cerebral Venous Thrombosis Consortium; received research support from BMS/Pfizer, Boehringer-Ingelheim, Daiichi Sankyo, the European Union, the German Federal Joint Committee Innovation Fund, and the German Federal Ministry of Education and Research, Helena Laboratories and Werfen as well as speakers' honoraria/consulting fees from Alexion, AstraZeneca, Bayer, Boehringer-Ingelheim, BMS/Pfizer, Daiichi Sankyo, Portola and Werfen (all outside the submitted work). All other authors have no conflicts of interest to disclose.

Contributions

GU, KA and TB designed the study. AB, FH, AJ, JK, VI, UE and AN were responsible for the treatment of the patient. GU and KA collected and analyzed the clinical data. JZ and NW performed the experiments. GU, JZ, KA, NW, SP and TB analyzed the data, interpreted the results and wrote the manuscript. All authors read and approved the manuscript.

Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) and from the Herzstiftung to TB (BA5158/4 and TSG-Study). GU (CS 3014-0-0) and JZ (CS 3015-0-0) take part in the MINT-Clinician Scientist Program of the Medical Faculty Tübingen, funded by DFG (493665037).

Data-sharing statement

Data may be requested for academic collaboration from the corresponding author.

References

1. Ropper AH, Klein JP. Cerebral venous thrombosis. N Engl J Med. 2021;385(1):59-64.
2. Payne AB, Adamski A, Abe K, et al. Epidemiology of cerebral venous sinus thrombosis and cerebral venous sinus thrombosis with thrombocytopenia in the United States, 2018 and 2019. Res Pract Thromb Haemost. 2022;6(2):e12682.
3. Althaus K, Möller P, Uzun G, et al. Antibody-mediated procoagulant platelets in SARS-CoV-2-vaccination associated immune thrombotic thrombocytopenia. Haematologica. 2021;106(8):2170-2179.
4. Greinacher A, Thiele T, Warkentin TE, Weisser K, Kyrle PA, Eichinger S. Thrombotic thrombocytopenia after ChAdOx1 nCov-19 vaccination. N Engl J Med. 2021;384(22):2092-2101.
5. See I, Su JR, Lale A, et al. US case reports of cerebral venous sinus thrombosis with thrombocytopenia after Ad26.COV2.S vaccination, March 2 to April 21, 2021. JAMA. 2021;325(24):2448-2456.
6. Uzun G, Althaus K, Hammer S, et al. Diagnostic performance of a particle gel immunoassay in vaccine-induced immune thrombotic thrombocytopenia. Hamostaseologie. 2023;43(1):22-27.
7. Zlamal J, Singh A, Weich K, et al. Platelet phosphatidylserine is the critical mediator of thrombosis in heparin-induced thrombocytopenia. Haematologica. 108(10):2690-2702.
8. Wang JJ, Armour B, Chataway T, et al. Vaccine-induced immune thrombotic thrombocytopenia is mediated by a stereotyped clonotypic antibody. Blood. 2022;140(15):1738-1742.
9. Greinacher A, Langer F, Schonborn L, et al. Platelet-activating anti-PF4 antibodies mimic VITT antibodies in an unvaccinated patient with monoclonal gammopathy. Haematologica.

2022;107(5):1219-1221.
10. Warkentin TE, Baskin-Miller J, Raybould AL, et al. Adenovirus-associated thrombocytopenia, thrombosis, and VITT-like antibodies. N Engl J Med. 2023;389(6):574-577.
11. Greinacher A, Selleng K, Palankar R, et al. Insights in ChAdOx1 nCoV-19 vaccine-induced immune thrombotic thrombocytopenia. Blood. 2021;138(22):2256-2268.
12. Baker AT, Boyd RJ, Sarkar D, et al. ChAdOx1 interacts with CAR and PF4 with implications for thrombosis with thrombocytopenia syndrome. Sci Adv. 2021;7(49):eabl8213.
13. Perry RJ, Tamborska A, Singh B, et al. Cerebral venous thrombosis after vaccination against COVID-19 in the UK: a multicentre cohort study. Lancet. 2021;398(10306):1147-1156.
14. Singh A, Toma F, Uzun G, et al. The interaction between anti-PF4 antibodies and anticoagulants in vaccine-induced thrombotic thrombocytopenia. Blood. 2022;139(23):3430-3438.
15. Warkentin TE. High-dose intravenous immunoglobulin for the treatment and prevention of heparin-induced thrombocytopenia: a review. Exp Rev Hematol. 2019;12(8):685-698.
16. Uzun G, Althaus K, Singh A, et al. The use of IV immunoglobulin in the treatment of vaccine-induced immune thrombotic thrombocytopenia. Blood. 2021;138(11):992-996.