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Running title: Anti-PF4 antibodies in CVST after AV infection

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Authorship contributions

G.U., K.A., and T.B. designed the study. A.B., F.H., A.J., J.K., V.I., U.E., and A.N. were responsible for the treatment of the patient. G.U. and K.A. collected and analyzed the clinical data. J.Z. and N.W. performed the experiments. G.U., J.Z., K.A., N.W., S.P. and T.B. analyzed the data, interpreted the results and wrote the manuscript. All authors read and approved the manuscript.

Disclosure of conflict of interests

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Sankyo, European Union, German Federal Joint Committee Innovation Fund, and 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). Other authors declare no competing financial interests.

**Data sharing statement**

Data may be requested for academic collaboration from the corresponding author.
Cerebral venous sinus thrombosis (CVST) is a rare cerebrovascular disorder predominantly observed in adults, but can pose unique challenges in pediatric patients. The underlying etiologies of CVST are diverse, encompassing hypercoagulable states, infections, and trauma. While concomitant thrombocytopenia is uncommon in CVST cases, instances of CVST and thrombocytopenia have been reported following administration of adenoviral vector-based vaccines, ChAdOx1 nCoV-19 and Ad26.COV2.S. 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 one week after an adenovirus infection. Recurrent occlusion and profound thrombocytopenia, which responded to 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 (11 x 10^9/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 antibodies, 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 PCR. PCR for following viruses were negative: SARS-CoV-2, Influenza A, Influenza B, RSV, HMPV, Rhinovirus, HSV-1, HSV-2, Enterovirus, CMV, and EBV. 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 50 x 10⁹/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 (a total of 45g IVIG) on days 9 and 10, leading to a swift normalization of the platelet count (>100 x10⁹/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 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 IgG PF4/heparin EIA 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. The HIPA assay was negative with low concentration of heparin making heparin-induced thrombocytopenia (HIT) very unlikely (Figure 2A). In contrast, the modified HIPA was positive (platelet aggregation within 5 min in the presence of PF4 and within 15-35 min without exogenous PF4), which is a serological pattern that mimics VITT (Figure 2A). Antibody-induced procoagulant platelets, determined by expression of P-selectin and phosphatidylserine (PS) externalisation on the platelet surface, were analyzed using flow cytometer as previously described. Antibody-mediated procoagulant platelet formation was observed in the absence of heparin, 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 immunoglobulin (IVIG, 30 µg/ml), indicating FcγRIIA dependency (Figure 2B). Increased PS externalization can initiate plasmatic coagulation and subsequent increased thrombin generation on the platelet surface. 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). 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.
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. Recently, platelet-activating anti-PF4 antibodies were detected in an unvaccinated patient with monoclonal gammopathy and multiple thrombotic complications. Similar to our case, Warkentin et al. published very recently 2 cases (a pediatric and an adult patient) developing anti-PF4 antibodies almost one week after adenovirus infection. 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 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. 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. 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 \(^\text{13}\) and therapeutic dose heparin can disrupt the interaction between VITT antibodies and PF4 in vitro.\(^\text{14}\) 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\(^\text{15}\). IVIG mitigates platelet activation via competitive Fc\(\gamma\)RIIA binding.\(^\text{16}\) In VITT patients with CVST, IVIG therapy correlated with lower mortality.\(^\text{13}\) 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\(^\text{10}\) 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.
References
Figure Legends

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 labelled with relevant interventions. Abbreviations and normal range of laboratory parameters: platelet count (normal range: 150-450 x10^3/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%), intravenous immunoglobulin (IVIG), Enzyme immunoassay (EIA).

Figure 2. Patient sera induces platelet activation and procoagulant platelet phenotype.

(A) HIPA: Patient serum activated platelets with exogenous platelet factor 4 (PF4) in heparin induced platelet activation (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 HIT and a 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 analysed after incubation with patient’s sera. Where indicated, platelets were pre-treated with IV.3 (Fcγ receptor IIA blocking monoclonal antibody) or immunoglobulin (IVIG). Patient’s serum was tested with washed platelets from four healthy donors. Historical samples of patients with HIT (n=3) and
VITT (n=3) was also shown on the figure. Abbreviations: HC, healthy control; **p<0.01, ****p<0.0001.

**Figure 3. Patient sera induces thrombus formation *in vitro.*

Platelet rich plasma 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 labelled 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 IVIG (30 µg/ml). After the labelling 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/cm²) 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 20 µm.
Figure 1

C

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Days 1-14: Platelet count (10^3/μl)

- Day 4: Platelet transfusion
- Day 5: Thrombectomy
- Day 10: Retroperitoneal bleeding
- Day 15: Progressive subdural hematoma
- Day 20: PF4/heparin IgG EIA: positive (OD 2.7)
- Day 21: HIPA with PF4: positive
- Day 26: Enoxaparin

Day of hospitalization

- Day 0: Transfer to our hospital
- Day 1: IVIG
- Day 6: PF4/heparin IgG EIA: positive (OD 1.8)
- Day 7: HIPA with PF4: positive
Figure 3

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