Supplemental material

Methods:

Preparation of washed platelets

Whole blood from healthy donors was centrifuged at 120g for 20 minutes (min*) without break at room temperature (RT). The supernatant platelet rich plasma (PRP) was gently collected and immediately supplemented with apyrase (5 µL/mL PRP, [Sigma-Aldrich, St. Louis, USA]) and prewarmed anticoagulant-citrate dextrose solution A (ACD-A)(111 µL/mL PRP). Subsequently, platelets were separated from PRP via centrifugation (650g, 7 min*, RT, without brake), resuspended in 5 mL of wash-solution (modified Tyrode buffer: 5 mL bicarbonate buffer, 20 percent (%) bovine serum albumin, 10% glucose solution, 2.5 U/mL apyrase, 1 U/mL hirudin [Pentapharm, Basel, Swiss], pH 6.3) and allowed to rest for 15 min* at 37°C. Following a final centrifugation step (650g, 7 min*, RT, without brake) platelets were resuspended in 2 mL of resuspension-buffer (50 mL of modified Tyrode buffer, 0.5 mL of 1 mM MgCl2, 1 mL of 2 mM CaCl2, pH 7.2) and adjusted to $300x10^9$ /L after cell count measurement at a Cell-Dyn Ruby hematological analyzer (Abbott Park, Illinois, USA).

Testing for anti-platelet factor 4 (PF4)/heparin antibodies

A commercially available IgG-Enzym Immune assay (EIA) was used in accordance to manufacturer's instructions (Hyphen Biomed, Neuville-sur-Oise, France). Per manufacturer's recommendation, a sample was considered reactive if the optical density (OD) was ≥ 0.500. The ability of sera to activate platelets was tested using the functional assay heparin induced platelet aggregation assay (HIPA). In brief, serum was tested with washed platelets from four different healthy donors in the absence (buffer alone), in the presence of heparin (0.2 IU/mL and 100 IU/mL) and PF4 (25µg/mL [Chromatec, Greifswald, Germany]). Reactions were placed in microtiter wells containing spherical stir bars and stirred at approximately 500 revolutions per minute (rpm). Wells were examined optically at five-minutes interval for loss of turbidity. A serum was considered reactive (positive) if a shift from turbidity to transparency occurred within 30 min in at least two platelet suspensions. Observation time was 45 min. Each test included a diluted serum from a patient with heparin induced thrombocytopenia (HIT) as a weak positive control, collagen (5µg/mL [Collagen Horn, Takeda, Linz, Austria]) as strong positive control and a serum from a healthy donor as a negative control.

Assessment of antibody-mediated procoagulant platelets

To exclude non-specific effects like the activation of platelets via complement or non-specific immune complexes, all sera were heat-inactivated (56°C for 30 min*), followed by a sharp centrifugation step at 5,000g. The supernatant was collected. All experiments involving patients' sera were performed after incubation of 5 µL serum with 25 µL washed platelets ($7.5x10^6$) for 1.5 h* under rotating conditions at RT. When indicated, cell suspensions were preincubated in presence of PF4 (25 µg/ml). Afterwards, samples were washed once (7 min*, 650g, RT, without brake) and gently resuspended in 75 µL of phosphate-buffered saline (PBS, Biochrom, Berlin, Germany). Platelets were then stained with Annexin V-FITC and CD62-APC (Immunotools, Friesoythe Germany) and directly analyzed by flow cytometry (FC). As positive control, washed platelets were incubated with ionomycin (5µM, 15 min* at RT [Sigma-Aldrich, St. Louis, USA]) and thrombin receptor activating peptide TRAP-6 (10 µM, 30 min at RT [Hart Biologicals, Hartlepool, UK]). Test results were determined as fold increase of the percentage of double CD62p/Phosphatidylserine (PS) positive events in platelets upon incubation with patients' sera compared to cells incubated with healthy donors tested in parallel.

Results

Clinical and laboratory features of vaccine induced immune thrombotic thrombocytopenia (VITT)

We report 5 patients (3 female, 2 male) with a median age of 47 years (range: 20 and 57 years) who were referred to our hospitals with suspected thrombotic complications after first vaccination with ChAdOx1 nCoV-19.

Case #1: female, 47 y., Cerebral venous sinus thrombosis (CVST)

A 47-year old female patient presented with headache, dizziness, and nausea 7 days after the first vaccination with ChAdOx1 nCoV-19. Initial platelet count revealed 56x10^9/L. Because of sustaining vertigo and increasing headache patient was referred to the neurology department. Coagulation parameters on the following days revealed a slight increased INR with 1.3, aPTT was also prolonged with 35s. Fibrinogen was in a normal range with 263 mg/dL and D-dimer was increased with 9 µg/ml. After further decrease of platelet count, a severe thrombosis of the sagittal superior sinus and right transverse sinus was detected in computed tomography (CT) and magnetic resonance (MR) imaging. HIT testing revealed positive results in the EIA (OD 2.07) and in HIPA. Patient was treated with argatroban (1.5-2 aPTT prolongation) with IVIG for 3 days (total dose: 140 g). After IVIG therapy the platelet count increased to 201x10^9/L.´The patient deteriorated 4 days after initiation of agatroban, directly at the day of first infusion of IVIG and revealed clinical and radiological signs of malignant brain edema and congestive hemorrhage. Therefore, bilateral hemicraniectomy has to be performed to decrease intracranial pressure. Neurological symptoms began to recover and the sagittal superior sinus showed radiological signs of recanalization. At the time of the writing of this manuscript (4 weeks after hospital admission), the patient was still in rehabilitation with persisting global aphasia.

Case #2: female, 57 y., CSVT, hematoma

A 57-year old female patient admitted to her family doctor because of continuing headache 9 days after the first vaccination with ChAdOx1 nCoV-19. Blood analysis showed thrombocytopenia (27x10x^9/µL) and the patient was transferred to a university hospital. At admission, she had petechiae in the extremities and hematoma. Platelet count was 25 x10^9/L and D-dimer was 54 µg/ml. Fibrinogen decreased (50mg/dl). Cranial CT revealed a thrombosis of the left sigmoid sinus and bleeding into right occipitotemporal region. HIT testing revealed positive result in EIA (OD: 3.23) and in HIPA (positive buffer reaction and

positive in presence of low molecular weight heparin). Anticoagulation was initiated with argatroban and IVIG was administered (2x1 g/kg body weight). After the therapy, platelet count steadily increased and D-dimer reduced. The patient was discharged with a platelet count of 237 x 10^9/L and D-dimer of 1.6 µg/ml after 7 days of hospitalization. Anticoagulation was changed to apixaban at discharge. No further thrombosis was observed during hospitalization and three weeks of follow-up.

Case #3: female, 29y., CVST

A 29-year old female patient had recurrent headache 9 days after the first vaccination with ChAdOx1 nCoV-19. At admission, MRI showed no signs of CVST. Platelet count at admission was 53 x10^9/L. Coagulation parameters were abnormal (fibrinogen 274 mg/dL, INR 1.2, aPTT 23 s). D-dimer was strongly elevated (32 µg/ml). Initial HIT-Testing revealed a positive rapid assay. Anticoagulation was then initiated with argatroban in prophylactic doses combined by dexamethasone (40 mg). After four days, the patient suffered from imminent headache and aphasia. High titer PF4/heparin IgG antibodies were detected in the EIA (OD 3.07). HIPA was positive with a positive buffer reaction and positive in presence of low molecular weight heparin. A second MRI showed CVST and a large parenchymal hemorrhage in the left hemisphere. The patient was then treated with a combination of argatroban at therapeutic doses and IVIG (2x1 g/kg body weight; total dose: 95 g). Platelet count recovered within four days to 114x10^9/L. At day nine after CVST diagnosis and stable hemorrhage, anticoagulation was changed to dabigatran 2x150 mg. The patient had no risk factor for thromboembolism except oral contraception. She had also suffered from chronic autoimmune thyroiditis with normal thyroid function under replacement therapy. Follow-up MRI showed beginning of the resorption of the hematoma and partial recanalization of the CVST. The patient was discharged after 22 days of hospitalization.

Case #4: male, 53 y., pulmonary embolism (PE), deep vein thrombosis (DVT)

A 53-year old male was admitted to hospital 7 days after the first vaccination with ChAdOx1 nCoV-19. He introduced himself because of slight shortness of breath, petechiae on the legs. Initial platelet count after admission was 8x10^9/L. D-dimer (>35µg/mL) were elevated and with a fibrinogen of 126 mg/dL with aPTT and INR in a normal range, patient presented beginning signs of consumption. Physical examination and ultrasound revealed a thrombosis in the right thigh (femoral vein). A CT-scan revealed thrombosis in both femoral veins and central embolism in the right lung. HIT-antibodies revealed strong positive result in the EIA (3.18 OD) and a positive HIPA-result (activation with buffer and low molecular weight

heparin). IVIG therapy (1 g/kg body weight for two consecutive days; total dose: 200 g) and alternative anticoagulation with argatroban was initiated immediately after diagnosis of VITT. Anticoagulation was switched to apixaban PO when the platelet count had reached $50x10^9$/L. The patient was discharged on day 11 after admission with a platelet count of $80x10^9$/L. In a follow-up interview 1 months later, he did not report any signs or symptoms of bleeding or thrombosis. His platelet count was stable at 180.000/µL.

Case #5: male, 20 y, CVST, PE

A-20-years old male patient with a body mass index of 37 admitted to the emergency room with severe headache 8 days after vaccination with ChAdOx1 nCoV-19. At admission, platelet count was $60x10^9$/L and D-dimer level was 12.4 µg/mL. As there was no evidence of thrombosis in a cranial CT scan, prophylactic anticoagulation with apixaban (2x2.5 mg) was initiated. On day 4 he was referred to university hospital where cMRI revealed thrombosis of the right transverse sinus. Additionally he complained of new onset thoracic pain on admission. CT of the thorax showed bilateral pulmonary artery embolism and infarct pneumonia. The dose of apixaban was increased to 2x10 mg and IVIG (1g/kg continuous administration on two consecutive days) was administered. High titer PF4/heparin IgG antibodies were detected in the EIA (OD 3.36). Platelet count increased to 124 $x10^9$/L. After 3 days, platelet count dropped again to 81 $x10^9$/L. Anticoagulation was changed to argatroban and a second dose of IVIG (1g/kg continuous administration on three consecutive days) was administered. A control MRI on the day of second IVIG administration showed progression of thrombus in size in right transverse sinus. After one week, MRI showed the regression of thrombosis in the transverse sinus. The patient received a total of 600 g IVIG. After IVIG therapy, platelet count increased to 175 $x10^9$/L. On 20^{th} day of the hospitalization, the anticoagulation was changed to dabigatran (2x150 mg). The patient was discharged after 22 days of hospitalization. The patient did not develop any further thrombosis during hospitalization and 3 weeks follow-up.

Supplemental Tables

Table 1A Demographic and clinical data of cases with vaccine induced immune thrombotic thrombocytopenia

Case #	Age	Sex	First symptoms after vaccination (days)	Thrombosis /Bleeding	Laboratory investigations at admission				
					PLT, (150-450 x109/L)	D-Dimer, (<0.5 µg/ml)	Fibrinogen, (170-410 mg/dl)	INR	aPTT, (>40s)
1	47	f	7	CVST	56	9	263	1.3	35
2	57	f	9	CVST, hematoma	27	54	50	1.5	21
3	29	f	9	CVST	53	32	274	1.2	23
4	53	m	7	DVT, PE	8	>35	126	1.0	25
5	20	m	8	CVST, PE	60	12.4	n.a.	1.1	33

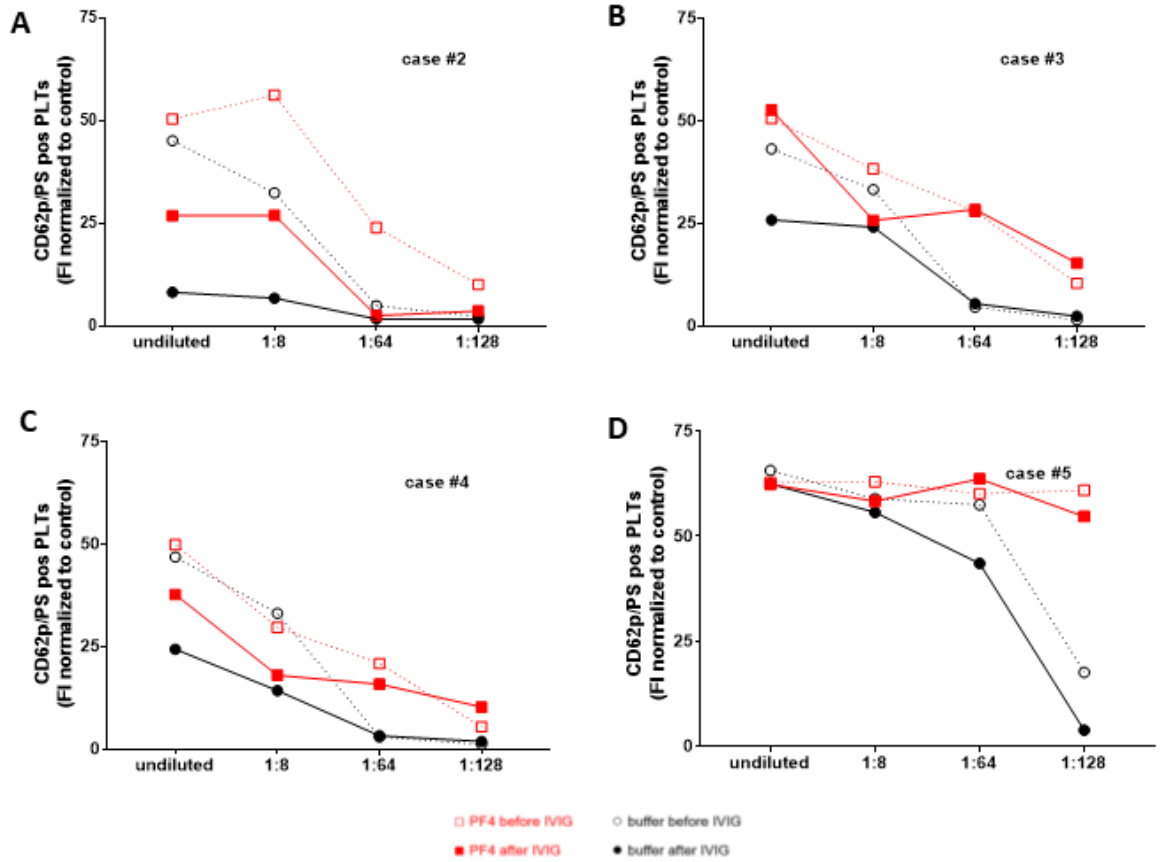
CVST indicates Cerebral venous sinus thrombosis; DVT, deep vein thrombosis; PE, pulmonary embolism; PLT, platelet, *upper limit of the D-dimer test; n.a., not available;

Table 1B Serological response to IVIG in VITT patients

Case #	Findings at admission			Time after IVIG therapy (day)	Findings after IVIG		
	Anti-PF4/heparin EIA (OD)	Modified HIPA (PF4) [time to aggregation, cutoff 30 min]	Procoagulant PLT [CD62p/PS pos PLTs, FI normalized to control, cut off 1.0]		Anti-PF4/heparin EIA (OD)	Modified HIPA (PF4) [time to aggregation, cutoff 30 min]	Procoagulant PLT [CD62p/PS pos PLTs, FI normalized to control, cut off 1.0]
1	2.07	5	23	/	n.a.	n.a.	n.a.
2	3.23	5	45	7	3.10	15	8
3	3.07	5	43	2	3.08	30	26
4	3.18	5	47	5	3.43	5	24
5	3.36	5	66	1	3.11	5	62

PF4 indicates platelet factor 4; EIA, enzyme immunoassay; OD, optical density; HIPA, heparin induced platelet activation assay; PLT, platelet; IVIG, intravenous immunoglobulin; n.a., not available

Supplemental Figure 1



Supplemental Figure 1: Effect of intravenous immunoglobulin (IVIG) therapy on procoagulant platelets

Procoagulant platelets (CD62P/Phosphatidylserine (PS) positive) were analysed in patients before and after IVIG therapy via Annexin V-FITC and CD62p-APC antibody staining. Where indicated, sera were serially diluted and PLTs were pre-treated with PF4 (Panel A-D). Data are presented as fold increase compared to healthy control.