Platelet activation and modulation in thrombosis with thrombocytopenia syndrome associated with ChAdOx1 nCov-19 vaccine

A severe clinical syndrome has been observed in some recipients of the ChAdOx1 nCov-19 or Ad26.COV2.S vaccine, characterized by the presence of antibodies against platelet factor 4 (PF4)/polyanions complexes, thrombocytopenia and thrombosis,[1-6] thus resembling heparin-induced thrombocytopenia (HIT).[1] The syndrome has been termed "thrombosis with thrombocytopenia syndrome (TTS)", or "vaccine-induced immune thrombotic thrombocytopenia (VITT)".[7,8] Intravenous immunoglobulin (IVIg) has been successfully used to increase the platelet count in patients with TTS.[3,4] Here we report on the management of two patients with TTS, the effect of their serum or plasma on normal platelets and its modulation by IVIg and anti-platelet agents. IVIg increased the platelet count and blunted the pro-thrombotic effect of sera and plasma from two patients with TTS, whereas IVIg and anti-platelets prevented *in vitro* TTS sera/plasma-supported thrombogenicity, platelet reactivity and markers of platelet activation.

Patient 1 is a 47 years old man who had an episode of syncope on March 15th 2021, 7 days after the first ChAdOx1 nCov-19 injection. He had thalassemia trait and had never been previously exposed to heparin. His platelet count was $92x10^9$/L at presentation and decreased to a nadir of $27x10^9$/L on day 4. A computed tomography angiography (CTA) detected pulmonary embolism, which was hemodynamically stable. Patient 2 is a 36 years old woman who experienced severe abdominal pain on March 17th 2021, 18 days after the first ChAdOx1 nCov-19 injection. She had never been previously exposed to heparin and never used oral contraceptives. Platelet count at presentation was $133x10^9$/L and decreased to a nadir of $106x10^9$/L on day 4. An abdomi-

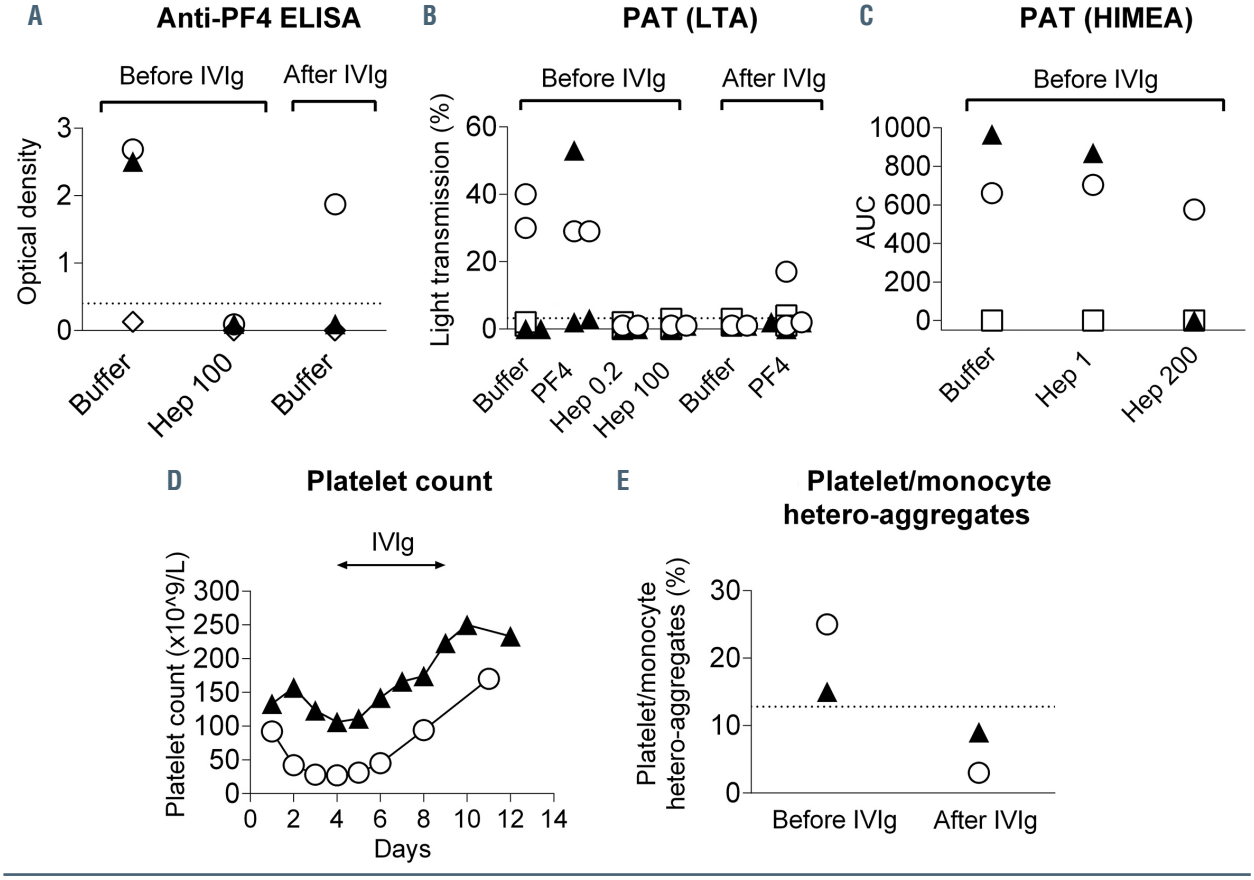

Figure 1. Immunologic tests and platelet parameters in patients before and after intravenous immunoglobulin administration and healthy subjects. Immunologic tests and platelet parameters in patients before and after intravenous immunoglobulin administration and healthy subjects. Blood withdrawal for all after intravenous immunoglobulin (IVIg) experiments was performed on day 15 for patient 1 and on day 13 for patient 2. Open squares: healthy subjects; open circles: patient 1; closed triangles: patient 2; open diamonds: patient 3 (post-vaccine thrombocytopenia without thrombosis). (A) Detection of anti-platelet factor 4 (PF4)/polyanions immunoglobulins by enzyme-linked immunosorbent assay (ELISA) in patients' sera in absence or presence of high concentrations of heparin (100 U/mL). The horizontal dotted line indicates the cut-off value of 0.4 optical density (O.D.) for normal values. (B) Platelet activation test (PAT), measured by light transmission aggregometry (LTA) in normal washed platelet suspensions (WPS). Serum samples (60 μL) from 7 healthy subjects and from patients 1 and 2 were added to 222 μL of normal WPS in a LTA aggregometer and platelet aggregation was measured as increase in light transmission for 30 minutes (min), in the absence and presence of low (0.2 U/mL) and high concentrations (100 U/mL) of heparin in 2 different experimental sessions, and in the presence of PF4 10 μg/mL in 2 (patient 1) and 3 (patient 2) experimental sessions. Individual results obtained in patients' sera and mean values obtained in sera from 7 healthy subjects are displayed. The horizontal dotted line indicates the cut-off value of 3.2% for normal values, which was calculated as mean + 2 standard deviations of results obtained in healthy subjects. (C) PAT, measured by impedance aggregometry (HIMEA) in normal whole blood (WB) samples. Serum samples (200 μL) from 1 healthy subject and from patients 1 and 2 were added to 300 μL of normal WB in a multiplate aggregometer and platelet aggregation was measured as area under the curve (AUC) for 15 min in the absence and presence of low (1.0 U/mL) and high concentrations (200 U/mL) of heparin. Sera from patients 1 and 2 were tested only before IVIg infusions. (D) Effects of IVIg infusion (2 gr/Kg body weight over 5 days) on platelet count in patient 1 and patient 2. (E) Percent of platelet/monocyte hetero-aggregates before and after IVIg infusion in patients 1 and 2. The horizontal dotted line indicates the cut-off value of 13.44% for normal values, which was calculated as mean + 2 standard deviations of results obtained with normal sera from 5 healthy subjects. Hep 0.2: heparin 0.2 U/mL; Hep 1: heparin 1 U/mL; Hep 100: heparin 100 U/mL; Hep 200: heparin 200 U/mL; PF4: platelet factor 4.

Table 1. *In vitro* effects of plasma or sera from patients or healthy subjects on parameters of platelet function in whole blood or washed platelet suspensions from healthy subjects.

Plasma* or serum* donor subjects	Thrombus formation in microchannels Surface coverage (%)	Thrombus formation in microchannels Mean thrombus area (μm^2)	PAT Light transmission (%)	Flow cytometry Platelets/monocytes heteroaggregates (%)	Flow cytometry Annexin V binding (%)
Healthy subjects	**5.35 ± 1.5**	**23.14 ± 5.3**	**1.86 ± 0.7**	**11.30 ± 0.1**	**0.70 ± 0.0**
(n. of subjects)	**(n=18)**	**(n=18)**	**(n=7)**	**(n=6)**	**(n=6)**
HS + Ig	3.66 ± 1.5	17.75 ± 4.2	2.0 ± 0	ND	ND
(n. of subjects)	(n=4)	(n=4)	(n=2)		
HS + aspirin	5.20 ± 2.2	16.75 ± 3.8	ND	ND	ND
(n. of subjects)	(n=4)	(n=4)			
HS + cangrelor	4.61 ± 1.36	18.75 ± 2.2	ND	ND	ND
(n. of subjects)	(n=4)	(n=4)			
Patient 1 before IVIg	**7.06 ± 3.7**	**24.67 ± 11.6**	**35 ± 13.8**	**22.06 ± 0.0**	**0.90 ± 0.0**
(n. of experiments)	**(n=3)**	**(n=3)**	**(n=7)**	**(n=2)**	**(n=2)**
Patient 1 before IVIg + Ig	2.97 ± 0.77	18.00 ± 6.1	1 ± 0	ND	ND
(n. of experiments)	(n=3)	(n=3)	(n=2)		
Patient 1 before IVIg + aspirin	7.25 ± 2.2	19.67 ± 4.7	1 ± 0	ND	ND
(n. of experiments)	(n=3)	(n=3)	(n=2)		
Patient 1 before IVIg + cangrelor	5.28 ± 2.4	18.67 ± 6.5	1 ± 0	ND	ND
(n. of experiments)	(n=3)	(n=3)	(n=2)		
Patient 1 after IVIg	5.40 ± 2.1	25.33 ± 11.7	1 ± 0	12.02 ± 0.1	0.77 ± 0.0
(n. of experiments)	(n=3)	(n=3)	(n=2)	(n=2)	(n=2)
Patient 2 before IVIg	**10.86 ± 2.1**	**34.33 ± 8.3**	**0 ± 0**	**86.68 ± 0.04**	**16.01 ± 0.1**
(n. of experiments)	**(n=3)**	**(n=3)**	**(n=2)**	**(n=2)**	**(n=2)**
Patient 2 before IVIg + Ig	5.45 ± 0.5	18.67 ± 1.5	ND	ND	ND
(n. of experiments)	(n=3)	(n=3)			
Patient 2 before IVIg + aspirin	9.48 ± 1.2	23.00 ± 1.7	ND	ND	ND
(n. of experiments)	(n=3)	(n=3)			
Patient 2 before IVIg + cangrelor	6.86 ± 2.7	21.33 ± 6.8	ND	ND	ND
(n. of experiments)	(n=3)	(n=3)			
Patient 2 after IVIg	7.17 ± 1.0	25.50 ± 6.1	1 ± 0	8.86 ± 0.0	0.55 ± 0.0
(n. of experiments)	(n=3)	(n=3)	(n=2)	(n=2)	(n=2)
Patient 3	**4.63 ± 0.4**	**19.0 ±1.4**	**ND**	**6.29 ± 0.0**	**0.51 ± 0.0**
(n. of experiments)	**(n=2)**	**(n=2)**		**(n=2)**	**(n=2)**

*Citrate plasma samples were used in experiments of thrombus formation in microchannels and of platelet activation test (PAT), while serum samples were used in flow cytometry experiments. Blood withdrawal for after-IVIg experiments was performed on day 15 for patient 1 and on day 13 for patient 2. Aspirin= 100 µmol/L; cangrelor= 1 µmol/L; Ig= 5 mg/mL. HS: healthy subjects; Ig:immunoglobulin; IVIg: intravenous immunoglobulin; pt: patient.

nal CT scan showed thrombosis of the portal, superior mesenteric and splenic veins, not associated with liver cirrhosis, occult malignancy or *JAK2* V617F. Both patients had normal platelet counts before vaccination.

Experiments for the confirmation of TTS diagnosis, the evaluation of platelet activation in such patients and its modulation by IVIg and anti-platelets were performed as follows. Anti-PF4/polyanions antibodies were measured by an enzyme-linked immunosorbent assay (ELISA, PF4 Enhanced Test, Immucor), which contains immunoglobulin G (IgG), IgA and IgM antibodies and is more sensitive than non-ELISA rapid immunoassays.[9] The platelet activation test (PAT) was measured (i) by light transmission aggregometry (LTA) using normal washed platelet suspensions (WPS) prepared by the method described by Mustard *et al.*[10] in the Platelet Aggregation Profiler-8E (Bio/Data, Milan, Italy), and (ii) by whole blood impedance aggregometry (HIMEA)[11] using normal whole blood (WB) in a Multiplate ECC (F. Hoffmann-La Roche). Platelets in WPS and WB were normally reactive to physiological agonists; patients' sera were tested in parallel in the same experimental sessions. For flow cytometry experiments, normal citrate-anticoagulated WB was incubated with anti-CD14-PE or annexin V-PE and anti-CD42b-FITC at room temperature (RT) for 20 minutes. Subsequently, samples for platelet-monocyte heteroaggregates were fixed, and red cells lysed. A total of 2,000 events of $CD14^+$ or 10,000 events of CD42b for annexin V were acquired at medium flow rate by FACS Verse Cytometer (BD Biosciences, San Jose, CA, USA). In some experiments, patients' sera were incubated with normal WB at RT for 20 minutes before staining. Experiments of *in vitro* thrombus formation were performed as previously described,[12] perfusing normal WB anticoagulated with lepirudin (450 ATU/mL) (Refludan, Pharmion) on collagen-coated (100 µg/mL) microchan-

nels at constant blood flow of 950/s shear rate for 4 minutes. Six images were then captured and the surface coverage and area of thrombi (ATh) were calculated. Ig (5 mg/mL) (Venital, Kedrion Biopharma), aspirin (100 μmol/L) (Sanofi SPA) or the P2Y12 antagonist cangrelor (1 μmol/L) (The Medicines Company, Parsippany-Troy Hills, NJ, USA) were added *in vitro* in some experiments.

The suspicion of TTS, based on the co-presence of thrombosis and thrombocytopenia, was supported by the positivity of the ELISA for anti-PF4/polyanions antibodies (Figure 1A), which was normalized by heparin at high concentration (100 U/mL). PAT was tested both by LTA and HIMEA after the addition of patients' sera to normal WPS and normal WB. Different results were obtained in the two patients: only serum from patient 1 induced aggregation of WPS, which was inhibited by heparin at low (0.2 U/mL) and high (100 U/mL) concentrations (Figure 1B); in contrast, both patients' sera induced platelet aggregation in normal WB, which was not inhibited by 1 U/mL heparin and was inhibited by 200 U/mL heparin only when induced by patient 2 serum (Figure 1C). The observed discrepant results obtained with WPS and WB might suggest a major role in patient 2 of leukocytes interaction with platelets and anti-PF4/polyanions autoantibodies in the pathogenesis of platelet activation and thrombosis.[13] As it has been demonstrated that the *in vitro* addition of PF4 increases the sensitivity of the PAT test in some patients, experiments were repeated in the presence of 10 μg/mL PF4 (Chromatec, Germany): under these conditions, serum from patient 1 induced platelet activation similarly in two separate experiments, while serum from patient 2 induced platelet activation in one experiment, but was still ineffective in two separate experiments (Figure 1B). Following the diagnosis of TTS, anticoagulant treatment, which was initially based on heparin preparations, was switched to alternative anticoagulants: fondaparinux during hospitalization and edoxaban at discharge for patient 1, argatroban and fondaparinux during hospitalization and apixaban at discharge for patient 2. Both patients were also treated with IVIg, 2 g/Kg body weight over 5 days, which normalized their platelet count (Figure 1D). The time needed to increase the platelet count was similar to that observed in other studies in which the same dose of IVIg was infused over 2 days.[3,4,14] No steroids were given to patients. Patient 2 also underwent transjugular intrahepatic portosystemic shunt (TIPS), thrombo-aspiration and loco-regional fibrinolysis in the angiography room on day 2. The clinical courses were uneventful for both patients, who were discharged on days 9 and 16. Platelet counts of both patients were normal up to 7 weeks after completion of IVIg treatment (*not shown*).

IVIg infusion had additional potentially protective effects: it i) reduced (patient 1) or normalized (patient 2) the serum reactivity detected by the ELISA test (Figure 1A), compatible with inhibition of antibody production;[15] ii) reduced or abolished the activation of normal WPS by patients' sera (Figure 1B); iii) normalized the percentage of circulating platelet/monocyte hetero-aggregates in both patients, a marker of platelet activation and interaction with leukocytes, which were increased at baseline (Figure 1E): similar findings were recently reported in other patients;[13] iv) blunted the amplifying effect of patients' sera on *in vitro* thrombus formation by normal blood (see below).

Considering that markers of platelet hyper-reactivity could be secondary to the patients' ongoing thrombotic process *in vivo* and that their improvement after IVIg could also be due to concomitant treatment with anticoagulants, we elected to evaluate the effects of patients' sera or plasma on markers of activation and reactivity of platelets from healthy subjects and the inhibitory effects of Ig added *in vitro*. To this end, the effects of patients' sera/plasma were compared not only to those of sera/plasma from six to 18 healthy subjects, but also to those of serum/plasma from a 76 years old man (patient 3) who developed thrombocytopenia ($69x10^9$/L) and epistaxis 6 days after the first ChAdOx1 nCov-19 injection, but had no thrombotic events and negative ELISA test results for anti-PF4/polyanions antibodies (Figure 1A). Compared to 18 plasma samples from healthy subjects, plasma from patients 2 and 1 (albeit less markedly), but not plasma from patient 3, increased thrombus formation by normal WB perfused over collagen-coated microchannels at 950/s shear rate (Table 1). Increased thrombus formation was not observed or was less marked with patients' plasma obtained after IVIg, and was completely prevented by Ig added *in vitro* (Table 1). Similar effects of patients' sera/plasma were observed on aggregation of normal WPS, formation of monocyte/platelet hetero-aggregates and binding of annexin V to procoagulant phosphatidylserine on the platelet membrane in normal WB, which were dramatically increased, especially by serum from patient 2. These effects were prevented by both the *in vivo* administration of IVIg and the *in vitro* addition of Ig, suggesting that Ig mostly inhibit platelet activation through FcγRIIa receptors, although a partial contribution by *in vivo* inhibition of antibody production cannot be ruled out.[15] Even though serum/plasma from patient 2 did not activate normal WPS, it was more pro-thrombogenic than serum/plasma from patient 1 in all other tests in which normal WB was used, thus reproducing the discrepant results obtained with WPS and WB in the PAT test (Figure 1B and C). Finally, we also tested the *in vitro* effects of the antiplatelet drugs aspirin and cangrelor on these parameters of platelet reactivity. Both drugs prevented the potentiation of platelet reactivity induced by patients' sera/plasma, although cangrelor tended to be more effective than aspirin. These results suggest that the thromboxane A2 and ADP/P2Y12 pathways of platelet activation might play a role in platelet activation in TTS. Whether or not these antiplatelet drugs could benefit TTS patients should only be determined by the results of *ad hoc* control studies.

In conclusion, we found that IVIg curbed the platelet-activating properties of our patients' sera and produced a lasting increase in platelet count even in absence of concomitant corticosteroid treatment.

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Contributions: MS and BC contributed to the design of the study, analyzed the data, contributed to writing the manuscript and critically reviewed it; MS, MC and CG performed laboratory analyses; TM and SB contributed to microfluidic device production and analysed thrombus formation data; BC and SB consulted on patient management; PV provided information regarding the negative control; MC and GMP designed the study, coordinated the group, contributed to data analysis and interpretation and wrote and edited the manuscript. All authors read and approved the final manuscript.

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Data sharing statement: the raw data that support the findings of this study will be made available by the authors, without undue reservation.

References

1. 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.
2. Scully M, Singh D, Lown R, et al. Pathologic antibodies to platelet factor 4 after ChAdOx1 nCoV-19 vaccination. N Engl J Med. 2021; 384(23):2202-2211.
3. Tiede A, Sachs UJ, Czwalinna A, et al. Prothrombotic immune thrombocytopenia after COVID-19 vaccine. Blood. 2021;138(4):350-353.
4. Thaler J, Ay C, Gleixner KV, et al. Successful treatment of vaccine-induced prothrombotic immune thrombocytopenia (VIPIT). J Thromb Haemost. 2021;19(7):1819-1822.
5. Vayne C, Rollin J, Gruel Y, et al. PF4 immunoassays in vaccine-induced thrombotic thrombocytopenia. N Engl J Med. 2021; 385(4):376-378.
6. 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.
7. Cattaneo M. Thrombosis with thrombocytopenia syndrome associated with viral vector COVID-19 vaccines. Eur J Intern Med. 2021; 89:22-24.
8. American Society of Hematology. Thrombosis with thrombocytopenia syndrome (also termed vaccine-induced thrombotic thrombocytopenia). Version 1.4; last updated April 29, 2021. https://www.hematology.org/covid-19/vaccine-induced-immune-thrombotic-thrombocytopenia. Accessed on May 1 , 2021.
9. Sachs UJ, Cooper N, Czwalinna A, et al. PF4-dependent immunoassays in patients with vaccine-induced immune thrombotic thrombocytopenia (VITT): results of an inter-laboratory comparison. Thromb Haemost. 2021 Jun 24. [Epub ahead of print]
10. Mustard JF, Perry DW, Ardlie NG, Packham MA. Preparation of suspensions of washed platelets from humans. Br J Haematol. 1972; 22(2):193-204.
11. Morel-Kopp MC, Mullier F, Gkalea V, et al; subcommittee on platelet immunology. Heparin-induced multi-electrode aggregometry method for heparin-induced thrombocytopenia testing: communication from the SSC of the ISTH. J Thromb Haemost. 2016 Dec;14(12):2548-2552.
12. Scavone M, Bozzi S, Mencarini T, Podda G, Cattaneo M, Redaelli A. Platelet adhesion and thrombus formation in microchannels: the effect of assay-dependent variables. Int J Mol Sci. 2020;21(3):750.
13. Greinacher A, Selleng K, Wesche J, et al. Towards understanding ChAdOx1 nCov-19 vaccine-induced immune thrombotic thrombocytopenia (VITT). https://www.researchsquare.com/article/rs-440461/v1. Accessed on April 30, 2021.
14. Uzun G, Althaus K, Singh A, et al. The use of intravenous immunoglobulin in the treatment of vaccine-induced immune thrombotic thrombocytopenia. Blood. 2021;138(11):992-996.
15. Galeotti C, Kaveri SV, Bayry J. IVIG-mediated effector functions in autoimmune and inflammatory diseases. Int Immunol. 2017; 29(11):491-498.