Vaccine-induced immune thrombotic thrombocytopenia: a possible pathogenic role of ChAdOx1 nCoV-19 vaccine-encoded soluble SARS-CoV-2 spike protein

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a rare catastrophic syndrome, occurring 5 to 30 days after the first dose of the adenoviral vector-based vaccines, ChAdOx1 nCoV-19 and coronavirus disease 2019 (COVID-19) Janssen vaccine, both encoding the full length severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) spike protein (SP).[1-3] Although VITT resembles autoimmune heparin-induced thrombotic thrombocytopenia, which is caused by the development of antibodies against platelet factor 4 (PF4)-polyanion complexes,[4,5] the etiopathogenesis, in particular what triggers the initial platelet activation, is still poorly understood. Recently published data indicate that ChAdOx1 nCoV-19 vaccine constituents[6] and the adenovirus *per se*[7] could be the polyanion that, binding to PF4, supports the formation of immunocomplexes which, in turn, activate platelets and stimulate the release of procoagulant neutrophil extracellular traps (NET).[6] The excipient EDTA in ChAdOx1 nCoV-19 vaccine has been shown to increase microvascular permeability, favoring dissemination of vaccine components into the bloodstream.[6] Interesting data from a preprinted paper show that adenoviral DNA can undergo alternative splicing, which could lead to the synthesis of soluble SP (sSP) variants lacking the membrane anchor but able to activate platelets and endothelial cells.[8] However, a case of VITT has been described after a second dose of the Moderna mRNA-based SARS-CoV-2 vaccine,[9] suggesting that other factors besides the adenoviral vector may be implicated in the pathogenesis of VITT.

In the present study, we tested the hypothesis that a sSP, possibly the product of alternative splicing, is the first trigger of VITT. The study was approved by the Ethics Committee of the University "La Sapienza" of Rome (study number 6305).

We studied three patients with VITT and seven vaccinated healthy controls within 3 weeks after the first dose of the ChAdOx1 nCoV-19 vaccine. The main demographics, clinical and laboratory findings of the studied cohort are summarized in Table 1.

VITT patients 1 and 2, both suffering from malignant middle cerebral artery stroke and extensive venous splanchnic and arterial pulmonary thrombosis, have been described previously.[10] Patient 3 was a 59-year-old male who developed complete intra-extrahepatic portal system and partial superior mesenteric vein thrombosis 11 days after vaccination. The diagnosis of VITT was based on the detection of high plasma levels of antibodies (IgG/IgM/IgA) against PF4-polyanion complexes, quantified by enzyme-linked immunosorbent assay (ELISA) (Immucor, Lifecodes, Waukesha, WI, USA), and positivity of the serum-induced platelet function test performed according to Guarino *et al.*[11] None of the volunteer healthy controls tested positive for anti-PF4 antibodies.

A hypercoagulable state was observed in VITT patients with high plasma levels of von Willebrand factor (VWF), D-dimer, and coagulation factor VIII. The reduction in factor XIII suggests an excessive consumption within thrombi. All VITT patients showed evidence of endothelial activation with significantly elevated VWF:RCo, the active form of VWF able to bind to platelet glycoproteins GPIb-IX-V (*Online Supplementary Figure S1*). Serum markers of NET, quantified by measuring DNA-myeloperoxidase complexes (Roche, cat. #11774425001) according to Kessenbrock *et al.*,[12] were elevated in all patients.

The thrombus retrieved from the middle cerebral artery of patient 1 was composed mainly of platelets and massively infiltrated by neutrophils[10] (*Online Supplementary Figure S2*). Most importantly we observed SP within the thrombus. Double staining with anti-CD61 (a platelet marker) and anti-SARS-CoV-2 SP antibodies showed co-localization of the two antigens. Immunohistochemistry, performed using two different antibodies: anti-angiotensin-converting enzyme 2 (mouse monoclonal anti-ACE2 - Cell Signaling Technology, Boston, MA, USA, cat. #74512, dilution 1:200) and anti-SARS-CoV-2 SP (rabbit polyclonal anti-SARS-CoV-2 SP - Cell Signaling Technology, Boston, MA, USA, cat. #56996, dilution 1:100), showed weak but diffuse SP staining which co-localized with ACE2 staining. A thrombus retrieved from a pre-pandemic age- and sex-matched stroke patient showed stronger diffuse staining for ACE2 but no evidence of staining for SP (Figure 1).

To test if signaling by sSP is related to the pathogenesis of VITT we investigated whether sSP and the soluble ACE2 (sACE2), the shed form of the primary receptor for SARS-CoV-2, were present in the serum of VITT patients by ELISA (My BioSource, San Diego, CA, USA, cat. #MBS7608267 and BioVision, Milpitas, CA, USA, cat. #E4528-100, respectively). All VITT serum samples and two of seven samples from volunteer healthy controls showed detectable levels of sSP and sACE2. Only one volunteer healthy control without evident sSP showed measurable sACE2. Serum from patient 1 was collected and analyzed at different intervals after the

Table 1. Summary of the demographics, clinical and laboratory data of patients with vaccine-induced immune thrombotic thrombocytopenia and of ChAdOx1 nCov-19 vaccinated healthy controls.

Subjects	Age (years)	Sex	Co-morbidities	Time from vaccine to symptoms (days)	D-dimer (µg/L) RV: 0-500	FBG mg/dL RV: 200-400	Platelets (x10^9/L) RV: 150-450	NET (ELISA)	Anti-PF4 (ELISA) RV: OD_{405} <0.5	Platelet activation assay (% ATP release) RV: <2%	sSP (pg/mL)	sACE2 (ng/mL)	Site of thrombosis	Treatment for VITT	Outcome
VITT Patient 1	57	F	HT Previous breast cancer	9	> 4318	366	44	0.231	Neg*	22	0.869	1.1929	Right MCA + PV+ Pulmonary arteries	IVIG Steroid Fondaparinux PE PLT transf.	Died
VITT Patient 2	55	F	HT	10	5441	336	133	1.696	1.29	14	0.868	2.843	Bilateral MCA + PV+ Pulmonary arteries	IVIG Steroid	Died
VITT Patient 3	59	M	HTN	9	> 4309	177	15	0.724	1.76	9	1.165	32.876	Intra- and extra-hepatic PV + Partial superior mesenteric vein	IVIG Steroid Fondaparinux PE PLT transf.	Recovering
	57 (median)			10 (median)											
V-HC 1	41	F	none	12	NA	NA	NA	NA	0.32	< 2%	0	0	NA	NA	-
V-HC 2	35	F	none	14	NA	NA	NA	NA	NA	NA	0	0.533	NA	NA	-
V-HC 3	35	F	none	12	NA	NA	NA	NA	0.09	< 2%	2.6	1.0094	NA	NA	-
V-HC 4	28	F	none	16	NA	NA	NA	NA	0.41	< 2%	1.88	2.561	NA	NA	-
V-HC 5	29	M	none	12	NA	NA	NA	NA	0.15	< 2%	0	0	NA	NA	-
V-HC 6	50	F	none	15	NA	NA	NA	NA	0.12	< 2%	0	0	NA	NA	-
V-HC 7	50	F	none	5	NA	NA	NA	NA	0.18	< 2%	0	0	NA	NA	-
	37 (median)			12 (median)											

Soluble SARS-CoV-2 spike protein, soluble ACE2, and anti-PF4 antibodies were assayed in serum collected within 72 h of hospital admission for patients with vaccine-induced immune thrombotic thrombocytopenia and within 16 days after the first dose of ChAdOx1 nCov-19 vaccination in healthy controls. *In patient 1, high levels of anti-PF4 antibodies were found at day 24 after vaccination (OD_{405}: 1.68). Platelet activation was not inhibited efficiently by high-dose heparin in patients 1 and 2 (ATP release after 20 min: 19% and 9%, respectively) whereas it was inhibited efficiently in patient 3 (ATP release after 20 min: 1%). RV: reference values; FBG: fibrinogen; NET: neutrophil extracellular traps; ELISA: enzyme-linked immunosorbent assay; OD_{405}: optical density; PF4: platelet factor 4; sSP: soluble spike protein; sACE2: soluble angiotensin-converting enzyme 2; VITT: vaccine-induced immune thrombotic thrombocytopenia; V-HC: vaccinated healthy control; F: female; M: male; HT: hypothyroidism; MCA: middle cerebral artery; PV: portal vein; IVIG: intravenous immunoglobulin (1 g/kg for 2 days); PE: plasma exchange; PLT transf.: platelet transfusion; HTN: hypertension; NA: not available. Value 0 means that the protein was not detected with this methodology.

stroke (*Online Supplementary Table S1*). Notably, sSP was still detectable at 35 days after vaccination with values comparable to those detected at days 9 and 11. Although a consistent reduction in ELISA reactivity for anti-PF4 antibodies was observed 20 days after plasma exchange, the platelet functional activity test showed that serum from VITT patient 1 was still able to induce platelet ATP release (20%) after 20 minutes suggesting that a stimulus other than anti-PF4 antibodies persisted in the blood and promoted platelet activation.

To evaluate whether the sSP detected in the serum of the VITT patients is implicated in platelet activation, serum obtained from VITT patients was used to stimulate washed platelets from three healthy donors in the absence or presence of an antibody directed against the S1 subunit of the SP (Invitrogen, clone P06DHuRb). For comparison we also tested the effect of a FcγIIa receptor-blocking antibody (Boster Biological Technology, clone IV.3) that blocks pla-

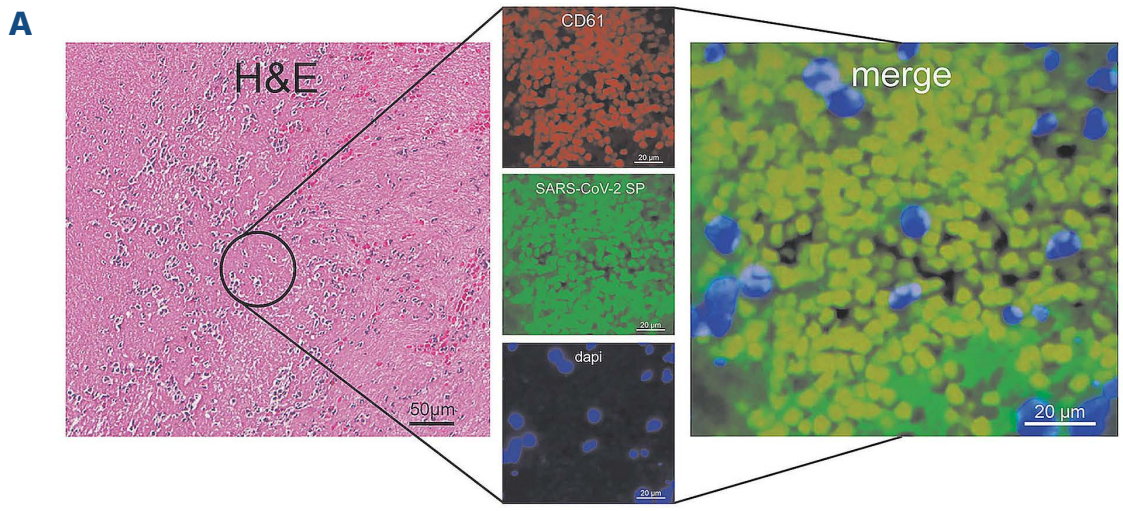

Figure 1. A thrombus from patient 1 with vaccine-induced immune thrombotic thrombocytopena was rich in platelets and stained positive for SARS-CoV-2 spike protein and ACE2. (A) Double immunofluorescence of thrombotic material retrieved from the right middle cerebral artery of patient 1 during the first mechanical thrombectomy. Staining with hematoxylin-eosin showed that the thrombus was made up almost exclusively of platelets, with abundant granulocytes. Platelets within the area encircled in (A) are stained in red with CD61 antibodies, the SARS-CoV-2 spike protein (SP) is stained in green, while the nuclei of the inflammatory cells are stained in blue with DAPI. The overlap of SARS-CoV-2 SP and platelets is shown in yellow (merge). (B) Immunohistochemistry highlights the presence of SARS-CoV-2 SP associated with decreased amounts of ACE2, within the thrombus of patient 1 (P1) as compared with a thrombus retrieved from a patient in the pre-pandemic era (CTL). H&E: hematoxylin-eosin; SARS-CoV-2: after severe acute respiratory virus coronavirus-2; ACE2: angiotensin-converting enzyme-2.

telet activation mediated by anti-PF4/polyanion complexes. After 15 minutes of incubation (at 37°C) with the respective antibodies, platelet activation was assessed by measuring the binding of PAC1-FITC (BD Bioscience, San Jose, CA, USA), which detects the active form of the platelet integrin αIIbβ3, and the binding of α-CD62P-PE, a marker of platelet degranulation, on a BD Accuri C6 flow cytometer. Sera from all VITT patients induced robust activation of platelets from healthy donors (Figure 2A, B). As expected, the anti-FcγRIIA blocking antibody inhibited the observed activation. Interestingly we also found that the antibody against the SP partially inhibited serum-induced platelet activation (Figure 2A, B); in detail, it significantly reduced the activation of the integrin by 60%, supporting our working hypothesis that SP may have a direct effect on platelet activation (Figure 2C).

During SARS-CoV-2 infection, SP can be shed and free S1 subunit can be released from both the virus and infected cells.[13] The concentration of SP in the plasma was shown to correlate with the severity of COVID-19. Although the presence of free S1 in the bloodstream could be the effect of tissue damage due to viral invasion, the potential, harmful effects of this circulating protein are still uncertain. Indeed, anomalous thrombogenic activity (with platelet activation and fibrinolytic impairment) has been observed in plasma from both COVID-19 patients and healthy individuals after SP addiction, suggesting a dangerous effect of SP *per se* on clot formation.[14]

The mechanism by which the SP could be triggering an effect on platelets is not known. Studies by Zhang and colleagues[15] have demonstrated that both SARS-CoV-2 and the SP alone can directly activate platelets via ACE2/SP interaction (although evidence of ACE2 receptors on platelets is controversial) and that platelet activation is suppressed by recombinant human ACE2 protein and by anti-SP monoclonal antibody. We found a partial suppression of platelet integrin activation in all VITT patients by using an antibody directed against the S1 subunit. Moreover, we observed that the level of ACE2 expression was lower on platelets inside the thrombus of a VITT patient than on platelets from the thrombus of a pre-pandemic control patient. A progressive reduction of ACE2 expression on platelets of critically ill COVID-19 patients and on platelets incubated with SARS-CoV-2 has been described. This suggests a possible internalization or shedding of the ACE2 receptor induced by SARS-CoV-2. A similar ACE2 downregulation could be hap-

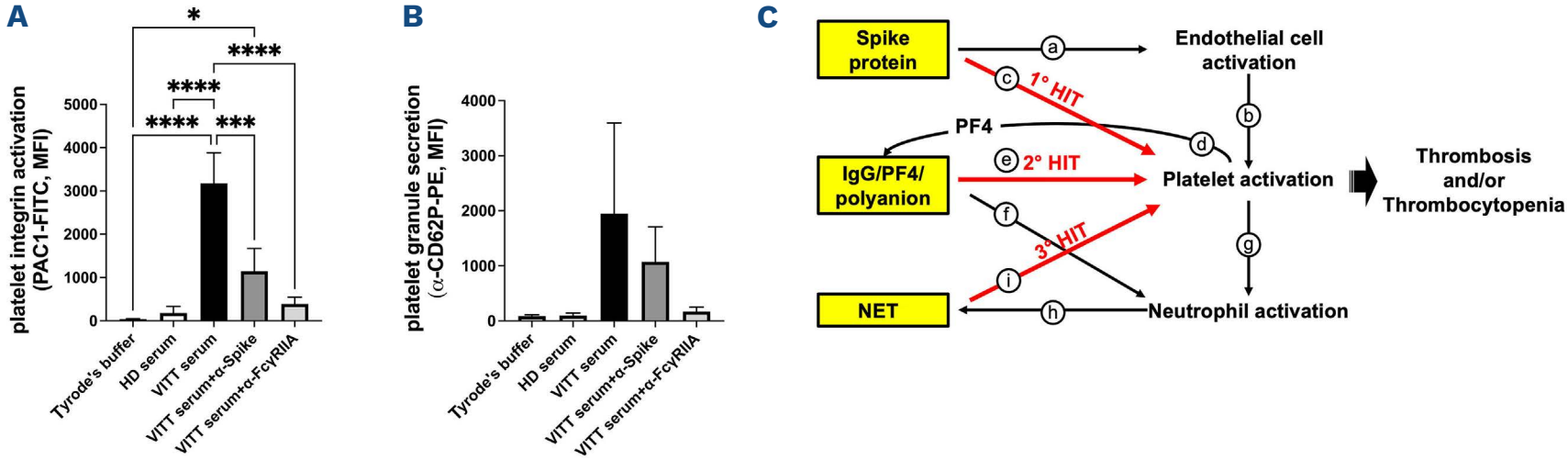

Figure 2. The SARS-CoV-2 spike protein contributes to ChAdOx1 nCoV-19 vaccine-induced platelet activation. (A, B) An antibody against the S1 domain of the SARS-CoV-2 spike protein (α-Spike) decreases vaccine-induced immune thrombotic thrombocytopenia (VITT) serum-induced platelet activation of washed platelets from healthy donors. Platelets were washed by serial centrifugation and resuspended in a solution of Tyrode's buffer and sera (3:1) at a final concentration of $5x10^7$ cells/mL. The blocking antibodies against the spike protein (SP) and FcγRIIA were incubated for 15 min at 4 μg/ml. Platelet activation was assessed by measuring (A) the binding of PAC1-FITC, an antibody that binds the active form integrin αIIbβ3, and (B) the binding of α-CD62P-PE, which is a marker of granule secretion, on a BD Accuri C6 Plus. The bar graph shows the mean ± standard deviation of the response of the platelets of the three patients (the response for each patient is the average of 3 technical replicates). In each experiment we always included negative controls with buffer alone and with sera from healthy donors who had been vaccinated with ChAdOx1 nCoV-19 but who did not experience any unusual side-effect after the injection. Statistical analyses were performed using ordinary one-way analysis of variance and the Hold-Sidak multiple comparison test. **P*<0.05; ***P*<0.01, ****P*<0.001, *****P*<0.0001. (C) Working model of the mechanism of vaccine-induced platelet activation. We postulate a multiple-hit model for platelet activation in the etiopathogenesis of VITT. The first hit is platelet activation by the SP. (a) The interaction between the SP and the ACE2 receptors on endothelial cells induces endothelial cell activation, (b) which results in platelet recruitment and activation through exposure of adhesion receptors and release of VWF. (c) The direct interaction between the SP and platelets would also activate platelets directly. (d) Activated platelets then release their granular contents which include large amounts of PF4 that, binding to polyanions, generate new antigens, which leads in some individuals to the production of anti-PF4/polyanion autoantibodies. (e) The second hit is the stimulation of platelets via the FcγRIIA by IgG/PF4 and IgG/PF4/polyanion immune-complexes. (f) IgG/PF4/polyanion immune-complexes also stimulate neutrophils that, (g) when co-stimulated by platelets, (h) release neutrophil extracellular traps (NET). (i) Thus, the third hit is NET, which support the coagulation cascade and further support platelet activation. These multiple stimuli amplify platelet activation and lead to thrombosis and/or thrombocytopenia. MFI: mean fluorescent intensity; HD: healthy donor.

pening in platelets of VITT patients, stimulated by the SP. Based on these preliminary findings we postulate a multiple-hit model for platelet activation in the etiopathogenesis of VITT (Figure 2C), which would explain why we could inhibit platelet activation by blocking either FcγRIIA or the sSP. The first hit would be the direct interaction between sSP and the ACE2 receptors on endothelial cells and, possibly, on platelets. Activated endothelial cells would induce platelet recruitment and adhesion by exposing adhesion receptors and releasing VWF. Activated platelets would release their granular contents, which include large amounts of PF4. Through the interaction of PF4 with polyanions, new antigens are generated with a consequent production of anti-PF4/polyanion autoantibodies. The second hit would be the stimulation of FcγRIIA by IgG/PF4 and IgG/PF4/polyanion immune-complexes resulting in the amplification of platelet activation. IgG/PF4/polyanion immune-complexes also stimulate neutrophils that, when co-stimulated by platelets, can release NET. Thus, the third hit is these traps, which support the coagulation cascade and further support platelet activation. Interestingly, patient 2 who had a rapidly fatal clinical course showed the highest level of NET.

We found SP in the serum of two volunteer healthy controls, but neither of them presented anti-PF4 antibodies, nor did their sera activate platelets from healthy donors. We can hypothesize that sSP variants are produced rarely from alternative nuclear splicing events but that, even more rarely, sSP variants capable of activating platelets can be transcribed. A lower ACE2 receptor expression on platelets, possibly genetically-determined, could also justify the different chance of platelets being activated by sSP. Experiments on more subjects, both vaccinated and non-vaccinated, and on VITT patients are mandatory to confirm the present data and our hypotheses.

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Disclosures

No conflicts of interest to disclose.

Contributions

MDM conceived and designed the study, enrolled the patients, interpreted the results, and prepared the original manuscript. PP, AC, and RR performed the ELISA on sera from all subjects included in this study, contributed to data interpretation and critically revised the manuscript. GD and ML performed the histopathological and immunohistochemical examinations of the retrieved thrombus and edited Figure 1. LS performed functional platelet activation assays, edited Figure 2 and critically revised the manuscript. FP performed the functional and serological platelet analyses and contributed to the interpretation of the results. AC is the consultant hematologist who made treatment decisions regarding the VITT patients and analyzed coagulation parameters. MI is the interventional neuroradiologist who retrieved the thrombus from patient 1, reviewed the manuscript and edited the figures and references. OGS, IB, EN, LP and MTD participated in data collection, edited the tables and revised the manuscript. DT critically reviewed and edited the manuscript.

Data-sharing statement

Data will be made available to researchers upon reasonable request.

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