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

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

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AUTHORS’ CONTRIBUTION

MDM conceived and designed the study, enrolled the patients, interpreted the results, and prepared the original manuscript. PP, AC, RR performed Elisa experiments 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 examination of the retrieved thrombus and edited Fig. 1. LS performed functional platelet activation assay, edited figure2 and critically revised the manuscript. FP performed the functional and serological platelets analysis and contributed to the interpretation of results; AC is the hematologist consultant who provided treatment decision making of VITT patients and performed coagulation parameters’ analysis; MI is the interventional neuroradiologist who retrieved the thrombus of patient 1, reviewed the manuscript and edited 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.

Disclosures: none.

Running head: Vaccine-induced immune thrombotic thrombocytopenia and spike protein

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DATA SHARING

Data will be made available to researchers upon reasonable request.

Word count: Main Text: 1500
Tables: 1; Figures: 2; Supplementary file: 1
References: 15
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 Covid-19 Janssen Vaccine, both encoding the full length SARS-coV-2 spike protein (SP). 

Although VITT resembles autoimmune heparin-induced thrombotic thrombocytopenia (HIT), due to the development of antibodies against platelet factor 4 (PF4)-polyanion complexes, the aetiopathogenesis and particularly what triggers the initial platelet activation is still poorly understood. Recently published data indicate that ChAdOx1 nCoV-19 vaccine constituents and the adenovirus per se 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 (NETs). The excipient EDTA in ChAdOx1 nCoV-19 vaccine has been shown to increase microvascular permeability, favoring dissemination of vaccine components into the bloodstream. Interesting data from a preprinted paper shows that the 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. However, a case of VITT has been described after a second dose of vaccine mRNA-based SARS-CoV-2 Moderna suggesting that other factors besides the adenoviral vector, may be implicated in VITT pathogenesis.

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 VITT patients and seven vaccinated healthy controls (V-HCs) within 3 weeks from the first dose of ChAdOx1 nCoV-19. 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 (MCA) stroke and extensive venous splanchnic and arterial pulmonary thrombosis, have been described previously.
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.

Diagnosis of VITT was based on the detection of high plasmatic levels of antibodies (IgG/IgM/IgA) against PF4-polyanion complexes, quantified by enzyme-linked immunosorbent assay (ELISA) (Immucor, Lifecodes, Waukesha, WI), and positivity of the serum-induced platelet function test performed according to Guarino et al.\textsuperscript{11} None of the V-HCs tested positive for anti-PF4 antibodies.

A hypercoagulable state was observed in VITT patients with high plasmatic levels of VWF, Ddimer, and coagulation Factor VIII. Factor XIII reduction 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 glycoprotein GPIb-IX-V (Online Supplementary Figure S1). Serum markers of NETs, quantified by measuring DNA-myeloperoxidase complexes (Roche, Cat. No: 11774425001) according to Kessenbrock et al.,\textsuperscript{12} were elevated in all patients.

The thrombus retrieved from patient 1 MCA was mainly composed of platelets and massively infiltrated by neutrophils\textsuperscript{10} (Online Supplementary Figure S2). Most importantly we observed the SP within the thrombus. Double staining with anti-CD61 (platelet marker) and anti-Sars-cov-2 SP antibodies showed co-localization of the two antigens. Immunohistochemistry, performed by using two different antibodies: anti-Angiotensin Converting Enzyme-2 (Mouse monoclonal anti-ACE2 - Cell Signaling Technology, Boston, MA, USA, cat.#74512, dil. 1:200) and anti-Sars-cov-2 SP (rabbit polyclonal anti-SARS-CoV-2 SP - Cell Signaling Technology, Boston, MA, USA, cat.#56996, dil. 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 a stronger diffuse staining for ACE2 but no evidence of staining for SP (Figure 1).

To test if signalling by the sSP is related to the pathogenesis of VITT we investigated whether sSP and the soluble ACE-2 (sACE2), the shed form of the primary receptor for Sars-cov-2, where
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 2 out of seven V-HCs showed detectable levels of sSP and sACE2. Only one V-HC without evident sSP showed measurable sACE2. Serum from Patient 1 was collected and analyzed at different post-stroke time intervals (Online Supplementary Table S1). Notably, sSP was still detectable at 35 days post-vaccination with values comparable to those detected at days 9 and 11. Notably, although a consistent reduction in ELISA reactivity for anti-PF4 antibodies was observed 20 days after plasma exchange, the platelet functional activity test still showed that the serum from VITT patient 1 was able to induce platelet ATP release (20%) after 20 minutes suggesting that in the blood a stimulus other than anti-PF4 antibodies persisted and promoted platelet activation.

To evaluate if 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 platelet activation mediated by anti-PF4/polyanion complexes. Following 15 minutes (37°C) of incubation with the respective antibodies, platelet activation was assessed by measuring the binding of PAC1-FITC (BD Bioscience, San Jose, California), that 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 a 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 inhibits serum-induced platelet activation (Figure 2A,B), in particular it significantly reduces 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 the infected cells \(^{13}\) and the SP concentration in the plasma were shown to correlate with COVID-19 disease severity. Although the presence of the 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 \textit{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 the 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. Moreover we observed that the ACE2 expression level was lower on platelets inside the thrombus of a VITT patient compared with 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 the 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 happening in platelets of VITT patients stimulated by the SP.

Based on these preliminary findings we postulate a multiple-hit model for platelet activation in VITT aetiopathogenesis (Figure 2C), which would explain why we could inhibit platelet activation either by blocking the FcγRIIA or the sSP. The first hit would be the direct interaction between the 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 includes large amounts of PF4. By 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 NETs. Thus, the third hit is NETs that support the coagulation cascade and further support platelet activation. Interestingly, patient 2 who had a rapidly fatal clinical course showed the highest level of NETs.

We found SP in serum of 2 V-HCs but neither of them presented anti-PF4 antibodies, nor their sera activated 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 to be activated by sSP. Experiments on more subjects, both vaccinated and non-vaccinated, and on VITT patients are mandatory to confirm present data and our hypotheses.

REFERENCES
Table 1: Demographics, clinical and laboratory summary of patients with VITT and of ChAdOx1 nCov-19 vaccinated healthy controls.

| Subjects | Age (median) | Sex | Comorbidities | Time from Vaccine to symptoms (days) | Ddimer (µg/L) RVs: 0-500 | FBG RVs: 200-400 mg/dL | PLT count (ELISA) RVs: 150,000-450,000 per mm³ | Anti-PF4 (ELISA) RVs: OD₄₀₅ <0.5 | Platelet activation assay (% ATP release) RVs: <2% | SP (pg/ml) RVs: 1.1929 | sACE2 (ng/ml) RVs: 0.869 | Site of Thrombosis | Treatment for VITT | Outcome |
|----------|--------------|-----|---------------|-------------------------------------|--------------------------|-------------------------|----------------------------------|----------------------------|---------------------------------|-----------------|-------------------|----------------|----------------------|---------|--------|
| VITT Patient 1 | 57 | F | HT | 9 | >4318 | 366 | 44,000 | 0.231 | Neg* | 22 | 0.869 | 1.1929 | Right MCA + PV + Pulmonary arteries | IVIG Steroid Fondaparinux PE PLT transf. | Fatal |
| VITT Patient 2 | 55 | F | HT | 10 | 5441 | 336 | 133,000 | 1.696 | 1.29 | 14 | 0.868 | 2.843 | Bilateral MCA + PV + Pulmonary arteries | IVIG Steroid | Fatal |
| VITT Patient 3 | 59 | M | HTN | 9 | >4309 | 177 | 15,000 | 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 |
| V-HCs 1 | 41 | F | none | 12 | NA | NA | NA | NA | 0.32 | <2% | 0 | 0 | NA | NA | - |
| V-HCs 2 | 35 | F | none | 14 | NA | NA | NA | NA | NA | 0 | 0.533 | NA | NA | - |
| V-HCs 3 | 35 | F | none | 12 | NA | NA | NA | NA | 0.09 | <2% | 2.6 | 1.0094 | NA | NA | - |
| V-HCs 4 | 28 | F | none | 16 | NA | NA | NA | NA | 0.41 | <2% | 1.88 | 2.561 | NA | NA | - |
| V-HCs 5 | 29 | M | none | 12 | NA | NA | NA | NA | 0.15 | <2% | 0 | 0 | NA | NA | - |
| V-HCs 6 | 50 | F | none | 15 | NA | NA | NA | NA | 0.12 | <2% | 0 | 0 | NA | NA | - |
| V-HCs 7 | 50 | F | none | 5 | NA | NA | NA | NA | 0.18 | <2% | 0 | 0 | NA | NA | - |
| V-HCs 8 | 37 | (median) | | 12 | (median) | | | | | | | | | | | |

**Table:** Table 1: Demographics, clinical and laboratory summary of patients with VITT and of ChAdOx1 nCov-19 vaccinated healthy controls.

**Subjects:** Includes patient demographics.

**Age:** Age of the patient in years.

**Sex:** Gender of the patient, either male (M) or female (F).

**Comorbidities:** Any pre-existing medical conditions.

**Time from Vaccine to symptoms (days):** Duration from vaccination to the onset of symptoms.

**Ddimer (µg/L):** D-dimer level in the blood.

**FBG:** Fasting blood glucose.

**PLT count (ELISA):** Platelet count as measured by ELISA.

**Anti-PF4 (ELISA):** Anti-PF4 antibody level.

**Platelet activation assay (% ATP release):** Percentage of ATP release.

**SP (pg/ml):** Serum protein level.

**sACE2 (ng/ml):** Serum ACE2 level.

**Site of Thrombosis:** Location of the blood clot.

**Treatment for VITT:** Medical treatments administered to manage VITT.

**Outcome:** Outcome of the patient's condition.
Soluble SARS-CoV-2 spike protein (SP), soluble ACE2 (sACE2), anti-PF4 antibodies were dosed in serum collected within 72 h from hospital admission for VITT patients and within 16 days from first dose of ChAdOx1 nCov-19 vaccination in healthy controls.

*In patient 1, high levels of anti-PF4 antibodies were found at day 24 from vaccination (1.68 OD405).

Platelet activation was not inhibited efficiently by high-dose heparin in patients 1 and 2 (percent of ATP release after 20 minutes of 19% and 9%, respectively) whereas it was inhibited efficiently in patient 3 (percent of ATP release after 20 minutes of 1%).

**Abbreviations:** RVs: Reference values; VITT: Vaccine-induced immune thrombotic thrombocytopenia; V-HCs: vaccinated healthy controls; HT: Hypothyroidism; HTN: Hypertension; IVIG: intravenous immunoglobulin (1 gr/kg for 2 days); FBG: fibrinogen; MCA: middle cerebral artery; NETs: Neutrophil Extracellular Traps; OD405: Optical Density; PV: portal vein; PE: plasma exchange; PLT: platelets; PLT transf.: platelets transfusion; NA: not available. Value 0 means that the protein has not been detectable with this methodology.
**FIGURE LEGENDS**

**Figure 1. Thrombus from VITT patient 1 was rich in platelets and stained positive for Sars-cov-2 Spike protein (SP) and ACE2**

A. Double immunofluorescence of thrombotic material retrieved from right middle cerebral artery of Patient 1 during the first mechanical thrombectomy. On hematoxylin-eosin stain (H&E), the thrombus was made up almost exclusively by platelets, with abundant granulocytes. Platelets within the area encircled in A are stained in red with CD61 antibodies, the SARS-CoV-2 SP is stained in green, while the nuclei of the inflammatory cells are stained in blue with DAPI. 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).

**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 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 5x10⁷ cells/ml. The blocking antibodies against the spike protein (SP) and the FcγRIIA were incubated for 15 minutes at 4µg/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 that is a marker of granule secretion, on a BD Accuri C6 Plus. The bar graph shows the mean ± SD of the response of the platelets of the three patients (the response for each patient is the average of the 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 did not experience any unusual side-effect after the injection.
Statistical analyses were performed using ordinary one-way ANOVA and 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 VITT aetiopathogenesis. 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) that 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 includes 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 NETs. (i) Thus, the third hit is NETs that support the coagulation cascade and further support platelet activation. These multiple stimuli amplify platelet activation and lead to thrombosis and or thrombocytopenia.
Supplementary Information

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to:

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

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The PDF file includes:

1. Supplementary Figure 1 (Figure S1)………………………….page 3
2. Supplementary Figure 2 (Figure S2)………………………….page 4
3. Supplementary Table 1 (Table S1)……………………………..page 5-6
Figure S1. Coagulation is activated in VITT patients compared to vaccinated healthy controls.

Factor VIII and XIII were respectively significantly increased and decreased in VITT patients within 72 h from hospital admission compared to vaccinated healthy controls (V-HC). All VITT patients showed evidence of endothelial activation with significantly elevated VWF:RCo compared to V-HC. Statistical analyses were performed using unpaired Student’s test. *P < 0.05; **P < 0.01.
Figure S2. Macroscopic aspect of the «white» (platelet-rich) thrombus retrieved during the first endovascular thrombectomy from middle cerebral artery in Patient 1. Thrombus length: 4 mm.
Table S1. Patient 1 laboratory findings over time, during her hospital stay. Note that soluble SARS-CoV-2 Spike protein (SP) was found up to 35 days after vaccination. Anti-PF4 antibodies were detected for the first time only at day 15 from admission. On day 45, despite negativity of anti-PF4 antibodies, serum patient continued to activate platelets from healthy donors and this activation was inhibited by both low and high dose of heparin (6% and 3% respectively). Platelet count reached normal values on day 12 after admission, 3 days after intravenous high dose immunoglobulin administration but decreased short after. Plasma exchange on 3 consecutively days

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*Note that soluble SARS-CoV-2 Spike protein (SP) was found up to 35 days after vaccination. Anti-PF4 antibodies were detected for the first time only at day 15 from admission. On day 45, despite negativity of anti-PF4 antibodies, serum patient continued to activate platelets from healthy donors and this activation was inhibited by both low and high dose of heparin (6% and 3% respectively). Platelet count reached normal values on day 12 after admission, 3 days after intravenous high dose immunoglobulin administration but decreased short after. Plasma exchange on 3 consecutively days.*
(3 l exchange with 5 % albumin replacement) was performed on day 15th from admission with a low response on PLT count. PLT: platelet; NETs: Neutrophil Extracellular Traps. NA: not available

*D-dimer was dosed by Emergency Department laboratory with a cut-off of 4318 μg/L except on day 12th when D-dimer was dosed by Hematology Laboratory with no cut-off.