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TO THE EDITOR:

Antiplatelet drugs block platelet activation by VITT patient serum

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Vaccines are an important part of the response to the SARS-COV-2 global pandemic. Although rare, aggressive thrombotic events at unusual sites, with accompanying thrombocytopenia and bleeding with high mortality, have increasingly been reported in young, healthy individuals at 4 to 30 days after vaccination with the Oxford-AstraZeneca chimpanzee adenovirus-vectored ChAdOx1 nCoV-19 (AZD1222).[1,2] This syndrome of vaccine-induced immune thrombocytopenia and thrombosis (VITT) clinically resembles autoimmune heparin-induced thrombocytopenia (HIT), in which antibodies against platelet factor 4 (PF4) bind and cross-link to the platelet surface receptor FcγRIIA (CD32a), inducing platelet activation.[1-3] VITT after the first AZD1222 vaccination has a reported incidence of between 1 in 25 000 and 1 in 100 000.[2,4,5]

In this study, we investigated the effect of serum from patients with VITT on platelet activation monitored by light transmission aggregometry (LTA), assessing the ability of clinically available antiplatelet drugs and kinase inhibitors to prevent platelet aggregation in vitro. Blood collection from patients, healthy individuals after AZD1222 vaccination, and nonvaccinated healthy donors were authorized under research ethics approvals 15/NW/0079 and 20/HRA/1817 and Birmingham University Internal Ethical Review approval ERN_11-0175, respectively. Experimental procedures are detailed in the supplemental Information (available on the *Blood* Web site).

Patients (or their next of kin in the case of those patients who lacked capacity) gave informed consent for collection of their blood in line with ethical principles laid out in the Declaration of Helsinki.

The presentations of 7 patients with VITT are summarized in Table 1. All patients were Caucasian and under the age of 50 with no previous symptomatic COVID-19. Patients presented with thrombosis (6 patients with cerebral venous sinus thrombosis [CVST] and 1 patient with ischemic stroke) and thrombocytopenia 9 to 14 days after the first AZD1222 vaccination. Clinical investigation at the time of presentation revealed all patients had thrombocytopenia (range, 7-113 $\times$ 10^9 platelets per L), with massively elevated D-dimer (range, 6574-62 342 ng/mL) and low fibrinogen (range, <0.35-2.36 g/L) levels. Despite no prior heparin exposure, HIT screening (anti-PF4 IgG Immucor enzyme-linked immunosorbent assay) showed strong reactivity in all patients. Heparin-induced platelet activation (HIPA) assays in the 4 patients tested showed activation in response to patient serum that was reduced by low heparin concentrations and blocked by high ones. Similar findings are reported in other patients with VITT.[1,2] All patients received IVIg and the steroid dexamethasone, as recommended by VITT treatment guidelines,[6] and 2 patients received plasma exchange. Platelet counts improved over 1 to 4 days in all patients except 1 who died 24 hours after presentation. At the time of this writing, 3 patients had recovered and been discharged from the hospital with ongoing normal platelet counts, 1 patient remained in hospital, and 2 patients had died because of the sequelae of CVST and secondary intracerebral hemorrhage. In addition, 1 discharged patient,

Table 1. Summary of clinical characteristics of patients with VITT

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age, y	48	32	21	46	43	44	42
Sex	Male	Female	Male	Female	Female	Male	Male
Platelet count at presentation ($\times 10^9$/L); normal range, 150-450	16	98	113	7	11	35	21
D-dimer at presentation (ng/mL); normal range, 0-250	62 342	6574	22 903	31 301	30 324	6807	27 000
Fibrinogen at presentation (g/L); normal range, 1.5-4	1.2	<0.35	0.98	1.1	1.07	<0.35	2.36
PT ratio at presentation; normal range, 0.8-1.2	1.2	1.5	1.3	1.2	1.1	1.4	1.1
APTT ratio at presentation; normal range, 0.8-1.2	1	1.7	0.8	1.1	1.2	1.6	1.3
HIT antibody screen at presentation (optical density); normal range, 0.01-0.4	2.45	2.17	2.8	>3.0	1.77	2.6	>3.0
Heparin-induced platelet activation (HITAlert) at presentation							
Platelet activation with serum, normal ≤8%	24.79%	31.2%	55%	N/A (not done)	N/A (not done)	N/A (not done)	75.31%
Platelet activation with serum and heparin, normal ≤8%	18.53%	18%	36.5%	N/A (not done)	N/A (not done)	N/A (not done)	22.63%
Platelet activation with serum and excess heparin, normal ≤8%	0.64%	3.68%	1.43%	N/A (not done)	N/A (not done)	N/A (not done)	4.38%
Clinical	CVST	CVST	Ischemic stroke	CVST	CVST	CVST	CVST
Days after vaccine at presentation	14	12	10	14	11	9	12

APTT, activated partial thromboplastin time; ICA, internal carotid artery; IVIg, intravenous immunoglobulin; N/A, not available; PEX, plasma exchange; PT, prothrombin time; SC, subcutaneous.

Table 1. continued

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Presentation symptoms	Headaches, hematuria, petechial rash; subsequent development of left-side weakness	Occipital headache	Headache for 2-3 d; collapse; expressive dysphasia	Headache	Headache, aura, petechial rash	Headache and vomiting for a few hours, followed by reduced conscious level	Headache for 1 wk, development of right-side weakness; subsequent seizure and collapse
Comorbidities	Prostatitis	None	None	Hypothyroidism; fibromyalgia; anxiety	None	None	None
Medications	None	None	None	Levothyroxine; sertraline; amitriptyline	None	None	None
Imaging findings at presentation	CVST; subarachnoid hemorrhage	CVST; subarachnoid hemorrhage; intraparenchymal hemorrhage	Acute left ICA thrombus with multiple left middle cerebral artery territory infarctions	CVST; intraparenchymal hemorrhage	CVST	CVST; left-side intracerebral hemorrhage; midline shift	CVST; subarachnoid and intraparenchymal hemorrhage; globalized brain atrophy
Immunosuppression regimen used	IVIg 0.5 g/kg once daily for 2 consecutive days; dexamethasone 20 mg once daily for 3 d	IVIg 1 g/kg for 2 consecutive days; dexamethasone 40 mg once daily for 4 d	IVIg 1 g/kg single dose; dexamethasone 40 mg once daily for 4 d	IVIg 1 g/kg single dose; dexamethasone 40 mg once daily for 4 d	IVIg 1 g/kg on 2 nonconsecutive days; dexamethasone 40 mg once daily for 3 d	IVIg 1 g/kg on 2 nonconsecutive days dexamethasone 40 mg once daily for 4 d	IVIg 1 g/kg single dose; dexamethasone 40 mg (2 doses)
Anticoagulant /antiplatelet regimen used	Argatroban fondaparinux 7.5 mg SC; once daily (when platelets normalized)	Argatroban	Fondaparinux 7.5 mg once daily; apixaban 5 mg twice daily (on discharge)	Fondaparinux 2.5 mg SC once daily (while platelets are $<50 \times 10^9$/L); fondaparinux 7.5 mg SC once daily (when platelets $\geq 50 \times 10^9$/L) dabigatran 150 mg twice daily (on discharge)	Fondaparinux 7.5 mg SC once daily	Argatroban fondaparinux 7.5 mg SC once daily (when platelets $\geq 050 \times 10^9$/L)	None
Other treatments required	Intubation	Intubation, thrombectomy	Thrombectomy	None	Plasma exchange	Intubation, thrombectomy, decompressive craniotomy, plasma exchange, platelet transfusion	Intubation, mannitol

APTT, activated partial thromboplastin time; ICA, internal carotid artery; IVIg, intravenous immunoglobulin; N/A, not available; PEX, plasma exchange; PT, prothrombin time; SC, subcutaneous.

Table 1. continued

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Timing of first serum sample	After IVIg and dexamethasone	After single dose of dexamethasone	Before treatment	Before treatment	After IVIg and dexamethasone	After IVIg and dexamethasone	Before treatment
Timing of second serum sample	N/A	Post -IVIg and dexamethasone	Post-IVIg and dexamethasone	Post-IVIg and dexamethasone	Post-PEX	Post-PEX	N/A
Days after IVIg that platelet count rose >50 × 10^9/L	N/A; nadir 59 × 10^9/L (platelets 100 × 10^9/L 2 d after first IVIg infusion).	2 d	N/A; nadir 52 × 10^9/L (platelets 198 × 10^9/L 3 d after IVIg infusion).	3 d	4 d	1 d	N/A; died <24 h after IVIg.
Outcome	Clinically recovered at time of discharge from hospital after a 26-d admission.	Death (support withdrawn after confirmation of brainstem death).	Clinically recovered at time of discharge from hospital after a 10-d admission; ongoing mild right hand weakness and expressive dysphasia.	Clinically recovered at time of discharge from hospital after a 16-d admission; 2 further admissions with headaches and drops in platelet counts; no further CVST; treated with (1st admission) prednisolone then (2nd admission) IVIg and rituximab; Remains in hospital.	Clinically recovered at time of discharge from hospital after a 12-d admission.	36-d intensive care admission; ventilator-associated pneumonia; Limited neurological recovery; remains in hospital.	Death 24 h after admission (rapid development of global ischemia before thrombectomy could be performed).

APTT, activated partial thromboplastin time; ICA, internal carotid artery; IVIg, intravenous immunoglobulin; N/A, not available; PEX, plasma exchange; PT, prothrombin time; SC, subcutaneous.

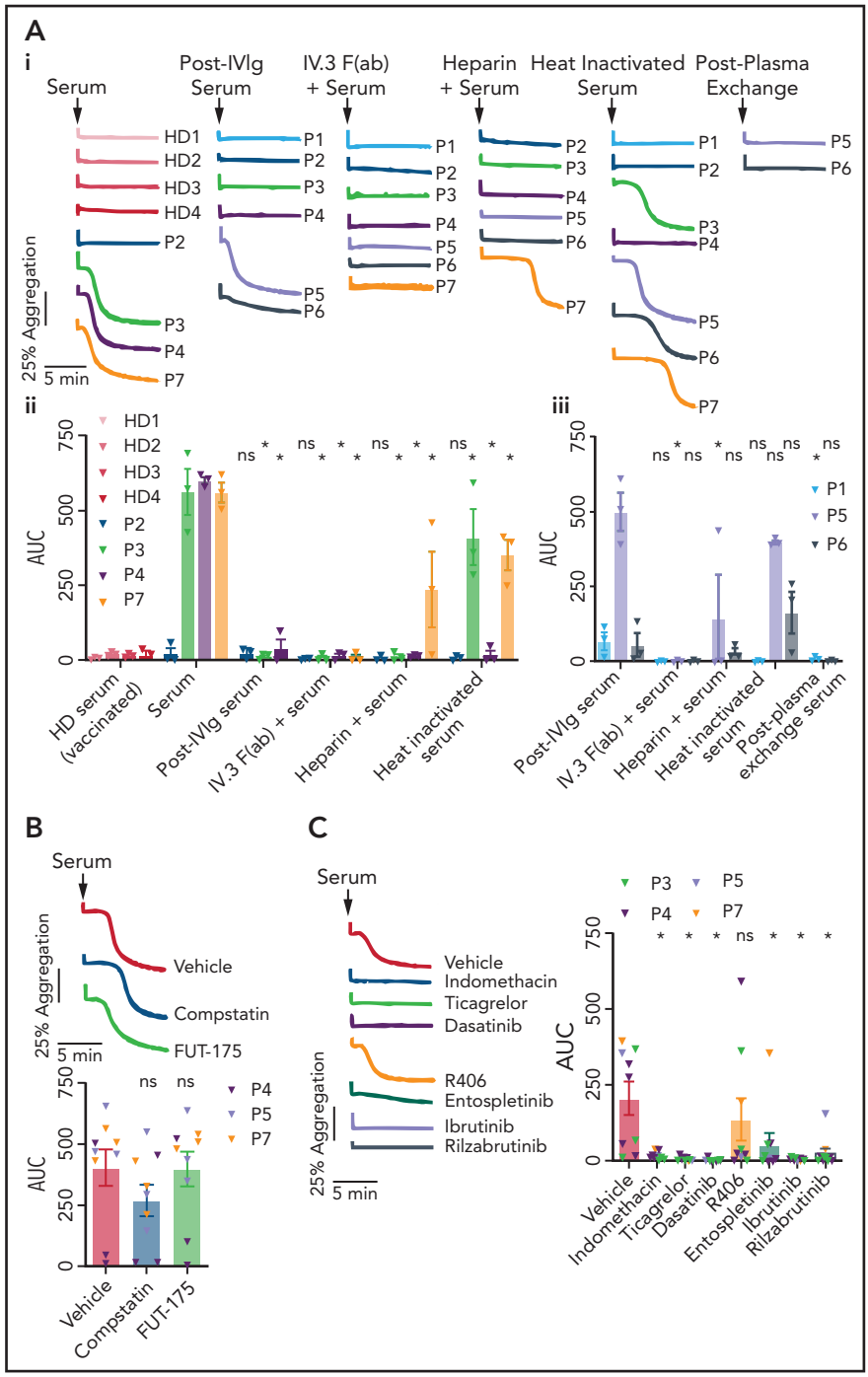

Figure 1. Serum from patients with VITT induces platelet aggregation via the FcγRIIA, and can be blocked by inhibition of COX, P2Y$_{12}$, Src, and Btk. Washed platelets (2 × 10^8/mL) were stimulated with serum (15:1, v/v), and aggregation was measured by light transmission aggregometry. (Ai) Representative aggregation traces for AZD1222-vaccinated healthy donors (HD) or patients with VITT (P) serum before and after IVIg treatment in the presence of Tyrode's buffer, 10 μg/mL IV.3 F(ab), low concentration heparin (0.2 U/mL), or after heat inactivation of complement (56°C, 45 minutes) and plasma exchange. Quantification of area under the curve (AUC) for 10 minutes for P2, P3, P4, P7 pre- and post-IVIg samples (Aii) and P1, P5, and P6 post-IVIg (Aiii) and plasma exchange samples. Mean ± standard error of the mean (SEM; n = 3). Statistical analysis was by 2-way analysis of variance (ANOVA) with Dunnett's multiple comparisons (vs serum [Ai]); vs post-IVIg serum [Aiii]), *P < .05. ns, non-significant. (B) The effect of the complement inhibitors compstatin (28 μM), FUT-175 (10 μM), or vehicle on aggregation in response to serum from VITT-affected patients. Inhibitors were incubated for 10 minutes before stimulation. Representative aggregation traces and quantification of AUC for 10 minutes. Mean ± SEM (n = 9; 3 repeats P4, P5, and P7, respectively). Statistical analysis was by 1-way ANOVA with Dunnett's multiple comparisons. ns, non-significant. (C) The effect of antiplatelet drugs and tyrosine kinase inhibitors. The effect of indomethacin (10 μM), ticagrelor (1 μM), dasatinib (1 μM), R406 (1 μM), entospletinib (1 μM), ibrutinib (0.5 μM), rilzabrutinib (0.5 μM) or vehicle (0.02% DMSO) on aggregation in response to VITT-affected patient serum. Inhibitors were incubated for 10 minutes prior to stimulation. Representative aggregation traces and quantification of AUC for 10 minutes. Mean ± SEM (n = 9; 3 repeats P3, 4 repeats P4, and 1 repeat P5 and P7). Statistical analysis was by 1-way ANOVA with Dunnett's multiple comparisons. *P < .05. ns, non-significant.

who was taking dabigatran, relapsed with thrombocytopenia and headaches but without thrombosis or raised D-dimer <8 weeks after discharge and required repeat treatment with IVIg and corticosteroids.

Serum from patients with VITT, but not age-matched AZD1222-vaccinated or non-vaccinated healthy donors, induced platelet aggregation (Figure 1Ai-ii and data not shown). Variable degrees of platelet aggregation, depending on patient serum and platelet donor, were observed (Figure 1Ai-ii), which is similar to results in HIT and other VITT studies, with platelets from some healthy donors not responding.[1,7] Low-titer anti-PF4 antibodies have been shown to develop after vaccination in a small percentage of healthy individuals; however, they do not cause platelet activation.[8] Aggregation was blocked after IVIg treatment, except in the 2 patients who did not clinically respond to IVIg and required plasma exchange (Figure 1Aii-iii). In these 2 patients, aggregation responses were blocked after plasma exchange (Figure 1Aii-iii). Eptifibatide treatment confirmed that responses were aggregation not agglutination (data not shown).

Platelet activation by patient serum was abolished by IV.3 F(ab) blockade of FcγRIIA (Figure 1Ai-iii). This result is similar to those in another report[1] and implies that activation is most likely mediated by clustering of the receptor by IgG and immune complexes,[9] demonstrating that platelet activation in VITT is mediated by FcγRIIA. Low concentrations of heparin are known to enhance platelet responses in HIT assays, whereas high concentrations are inhibitory.[10,11] In contrast, low (0.2 U/mL) concentrations of heparin prevented (5 of 7 patients) or delayed (2 of 7 patients) aggregation (Figure 1Ai-iii). High heparin concentration (100 U/mL) blocked aggregation (data not shown).

Immune complexes that activate platelets via FcγRIIA have been reported in patients critically ill with COVID-19.[12] In these patients, who had been exposed to heparin and displayed thrombocytopenia and thrombosis, HIT was ruled out, because of the lack of anti-PF4 antibodies and platelet activation independent of heparin.[12] Analogous to our findings, platelet activation by these immune complexes was blocked by both low and high concentrations of heparin.[12] Our observation that heparin blocks platelet aggregation, which is consistent with HIPA results and other reports,[1,13,14] implies that the decision to withhold heparin use in patients with VITT perhaps should be revisited. Unfractionated heparin treatment has been reported in 1 patient with VITT without deleterious effect.[14]

Anti-SARS-CoV-2 spike protein IgG antibodies from patients with severe COVID-19 have been shown to induce apoptosis and increase phosphatidylserine externalization in platelets mediated by FcγRIIA, although IgG aggregates or immune complexes could not be isolated from patient sera.[15] It is possible that a similar mechanism is occurring in patients with VITT. Activation of FcγRIIA could give rise to phosphatidylserine exposure and procoagulant platelets, which may lead to the extensive thrombosis and thrombocytopenia observed in patients with VITT.[13]

A role for complement has been proposed in VITT. Heat treatment of sera, which inactivates complement (56°C, 45 minutes), blocked aggregation in 3 of 7 patients (Figure 1Ai-iii), whereas minor effects on aggregation were observed with compstatin (a C3a inhibitor) and FUT-175 (a C3, C4, and C5 inhibitor; Figure 1B). These findings indicate that, although complement is not critical, it may reinforce platelet activation. Eculizumab (anti-C5 monoclonal antibody) treatment has been reported in 2 patients with VITT, in whom anticoagulation and IVIg or plasma exchange failed.[14] Both patients rapidly improved. The involvement of complement, which mediates a broad range of thromboinflammatory reactions involving endothelium, monocytes, and neutrophils, as well as platelets, in VITT pathology should be considered.[16] Normal serum complement levels in patients with VITT have been reported.[2]

We tested a variety of clinically used antiplatelet drugs and inhibitors of kinases downstream of FcγRIIA to determine whether they could prevent platelet aggregation in response to patient sera.[17] The COX inhibitor indomethacin, which works via the same mechanism as aspirin, and the $P2Y_{12}$ inhibitor ticagrelor prevented aggregation in response to patient serum, as did the Src inhibitor dasatinib and the Btk inhibitors ibrutinib and rilzabrutinib, with a significant reduction observed in response to the Syk inhibitor entospletinib (Figure 1C). This inhibition occurred irrespective of heterogeneity in samples from patients with VITT. All inhibitors were used at a concentration that fully inhibited aggregation in response to 3 μg/mL collagen (results not shown).

Although these antiplatelet and kinase inhibitors prevent aggregation in healthy donor platelets in vitro, further study in more physiological and clinically relevant assays assessing multiple additional readouts is needed before their use in treating patients with VITT can be considered. The potential clinical utility of some of these agents may be limited, however, by their associated bleeding risk. The risk of major bleeding with population-wide use of the COX inhibitor aspirin outweighs any theoretical benefit for this rare syndrome.[18] It should also be noted that VITT has been diagnosed in a patient already taking aspirin,[19] and our patient, who was initially treated with aspirin for a stroke, still developed progressive thrombocytopenia despite this intervention. Similarly, ticagrelor, dasatinib, and ibrutinib are associated with increased bleeding risk, so their use in patients with thrombocytopenia cannot be recommended.[20-22] Rilzabrutinib, currently in trials for immune thrombocytopenia (ITP) with no bleeding or thrombotic events reported,[23] appears to be a more promising treatment for further study, as does the Syk inhibitor fostamatinib, which is also an ITP treatment that lowers thrombosis without causing bleeding[24]; however, its active metabolite R406, used in this study at its clinically relevant concentration, did not effectively block platelet activation in vitro. Entospletinib, although not associated with bleeding, is not yet routinely used outside of clinical trials and has not been used in patients with thrombocytopenia.[25] If ongoing treatment is required because of inadequate response to the scarce and expensive IVIg and plasma exchange, then these antiplatelet agents have a potential role and warrant further evaluation.

The limitations of this study are the small sample size and the differing treatments received before collection of the patient samples. In addition, only a limited number of conditions were tested because of the volume of sera available, and aggregation

was measured only over a period of 10 minutes, with current consensus for examining aggregation in response to serum from patients with VITT for 30 minutes.

Overall, we have demonstrated that serum from patients with VITT, but not healthy AZ1222D-vaccinated donors, activates platelets via FcγRIIA, which can be blocked in vitro by antiplatelet therapies and tyrosine kinase inhibitors. Further assessment of these potential therapeutic interventions in physiological and clinically relevant models are needed before their use in patients with this rare syndrome can be considered.

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Authorship

Contribution: C.W.S. designed and performed the experiments, analyzed the data, and wrote and revised the manuscript; P.L.R.N. recruited the patients, designed and performed the experiments, analyzed the data, and wrote and revised the manuscript; S.J.M. performed the experiments and revised the manuscript; C.K. designed and performed the experiments and revised the manuscript; Y.D. generated reagents and revised the manuscript; S.P.W. revised the manuscript and designed the experiments; G.C.L. and W.A.L. recruited the patients, revised the manuscript, and contributed intellectually.

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Footnote

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