

Anti-platelet drugs block platelet activation by vaccine-induced immune thrombocytopenia and thrombosis patient serum

Smith, Christopher; Montague, Samantha; Kardeby, Caroline; Di, Ying; Lowe, Gillian; Lester, William; Watson, Steve; Nicolson, Pip

DOI:

[10.1182/blood.2021012277](https://doi.org/10.1182/blood.2021012277)

License:

None: All rights reserved

Document Version

Peer reviewed version

Citation for published version (Harvard):

Smith, C, Montague, S, Kardeby, C, Di, Y, Lowe, G, Lester, W, Watson, S & Nicolson, P 2021, 'Anti-platelet drugs block platelet activation by vaccine-induced immune thrombocytopenia and thrombosis patient serum', *Blood*. <https://doi.org/10.1182/blood.2021012277>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

This research was originally published in Blood Online. Christopher W Smith, Samantha Jayne Montague, Caroline Kardeby, Ying Di, Gillian C Lowe, William A Lester, Steve P Watson, Phillip Lindsay Ross Nicolson. Anti-Platelet Drugs Block Platelet Activation by Vaccine-Induced Immune Thrombocytopenia and Thrombosis Patient Serum. *Blood*. Prepublished August 10, 2021; DOI 10.1182/blood.2021012277.

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.



American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
editorial@hematology.org

Anti-Platelet Drugs Block Platelet Activation by Vaccine-Induced Immune Thrombocytopenia and Thrombosis Patient Serum

Tracking no: BLD-2021-012277R2

Christopher Smith (University of Birmingham, United Kingdom) Samantha Montague (University of Birmingham, United Kingdom) Caroline Kardeby (University of Birmingham, United Kingdom) Ying DI (University of Birmingham, United Kingdom) Gillian Lowe (University Hospital Birmingham NHS Foundation Trust, United Kingdom) William Lester (University Hospital NHS Foundation Trust Birmingham, United Kingdom) Steve Watson (University of Birmingham, United Kingdom) Phillip Nicolson (University Hospital Birmingham NHS Foundation Trust, United Kingdom)

Abstract:

Conflict of interest: COI declared - see note

COI notes: PLRN and SPW have received research grants from Novartis, Principia and Rigel Pharmaceuticals. PLRN has had honoraria from Bayer.

Preprint server: Yes; MedRxiv <https://doi.org/10.1101/2021.04.24.21255655>

Author contributions and disclosures: CWS and PLRN designed and performed experiments, analysed data and wrote and revised the manuscript. SJM performed experiments and revised the manuscript. CK designed and performed experiments and revised the manuscript. YD generated reagents and revised the manuscript. SPW revised the manuscript and designed experiments. GCL and WAL recruited patients, revised the manuscript and contributed intellectually.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: For original data, please contact corresponding author Dr Phillip L.R. Nicolson. Individual participant data will not be shared.

Clinical trial registration information (if any):

Anti-Platelet Drugs Block Platelet Activation by Vaccine-Induced Immune Thrombocytopenia and Thrombosis (VITT) Patient Serum

Christopher W. Smith,¹ Samantha J. Montague,¹ Caroline Kardeby,¹ Ying Di,¹ Gillian C. Lowe,² William A. Lester,² Steve P. Watson,¹ Phillip L.R. Nicolson,^{1,2}

¹ Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK

² Comprehensive Care Haemophilia Centre, University Hospitals Birmingham NHS Foundation Trust, Birmingham, B15 2TH, UK

Corresponding author: Phillip L.R. Nicolson, email: p.nicolson@bham.ac.uk

Manuscript word count: 1426

Number of figures: 1

Number of tables: 1

Number of references: 25

Short title: Blockade of Platelet Activation to VITT Patient Serum

Key points

- Serum from patients with VITT activates platelets via the FcγRIIA and can be blocked by COX, P2Y₁₂, Src, Syk and Btk inhibition.

Key words: VITT, FcγRIIA, AZD1222, COVID vaccination, Btk, PF4

To the Editor:

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 in young, healthy individuals 4–30 days after vaccination with the Oxford-AstraZeneca chimpanzee adenovirus vectored ChAdOx1 nCoV-19 (AZD1222) have increasingly been reported.^{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 the platelet surface receptor FcγRIIA (CD32a) inducing platelet activation.^{1–3} VITT following first AZD1222 vaccination has a reported incidence between 1 in 25,000 and 1 in 100,000.^{2,4,5}

In this study, we investigate the effect of serum from patients with VITT on platelet activation monitored by light transmission aggregometry (LTA), assessing the ability of clinically available anti-platelet drugs and kinase inhibitors to prevent platelet aggregation *in vitro*. Blood collection from patients, healthy individuals following AZD1222 vaccination and unvaccinated healthy donors were approved under

research ethics 15/NW/0079, 20/HRA/1817 and Birmingham University Internal Ethical Review (ERN_11-0175) respectively. Experimental procedures are detailed in Supplemental Data.

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 presentation of seven patients with VITT are summarized in Table 1. All patients were Caucasian, under the age of 50 with no previous symptomatic COVID-19. Patients presented with thrombosis (six patients: cerebral venous sinus thrombosis [CVST], one patient: ischemic stroke) and thrombocytopenia 9–14 days after first AZD1222 vaccination. Clinical investigation at the time of presentation revealed all patients were thrombocytopenic (range: $7-113 \times 10^9$ platelets/L), with massively elevated D-dimer (range: 6574-62342 ng/mL) and low fibrinogen (range: <0.35-2.36 g/L) levels. Despite no prior heparin exposure, HIT screening (anti-PF4 IgG Immucor ELISA assay) showed strong reactivity in all patients. Heparin Induced Platelet Activation (HIPA) assays in the four patients tested showed activation to patient serum which was reduced by low and blocked by high heparin concentrations. 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 two patients received plasma exchange. Platelet counts improved over 1-4 days in all patients except one who died 24 hours after presentation. At the time of writing, three patients had recovered and been discharged from hospital with ongoing normal platelet counts, one patient remains in hospital and two patients died because of the sequelae of CVST and secondary intracerebral haemorrhage. Additionally, one discharged patient, who was taking dabigatran, relapsed with thrombocytopenia and

headaches but without thrombosis or raised D-dimers less than 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 1A and data not shown). Variable degrees of platelet aggregation, depending patient serum and platelet donor, were observed (Figure 1A), which is similar to HIT and other VITT studies, with platelets from certain healthy donors not responding.^{1,7} Low titre anti-PF4 antibodies have been shown to develop following vaccination in a small percentage of healthy individuals, they however do not cause platelet activation.⁸ Aggregation was blocked post-IVIg treatment except in the two patients who did not clinically respond to IVIg and required plasma exchange (Figure 1A). In these two patients, aggregation responses were blocked post plasma exchange (Figure 1A). Eptifibatide treatment confirmed responses were aggregation not agglutination (data not shown).

Platelet activation by patient serum was abolished by IV.3 F(ab) blockade of FcγRIIA (Figure 1A). This is similar to other reports¹ and implies activation is likely mediated by clustering of the receptor by IgG and immune complexes,⁹ demonstrating platelet activation in VITT is mediated via 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 (5/7 patients) or delayed (2/7 patients) aggregation (Figure 1A). High heparin concentration (100 U/mL) blocked aggregation (data not shown).

Immune complexes that activate platelets via FcγRIIA have been reported in critically ill patients with COVID-19.¹² In these patients, who had been exposed to heparin and displayed thrombocytopenia and thrombosis, HIT was ruled out, due to lack of anti-

PF4 antibodies and platelet activation independent of heparin.¹² Analogous to our findings, platelet activation by these immune complexes could be 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 the decision to withhold heparin use in patients with VITT may need to be revisited. Unfractionated heparin treatment has been reported in one 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 phosphatidyl serine externalisation in platelets mediated by FcγRIIA, although IgG aggregates or immune complexes were not able to 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 phosphatidyl serine exposure and procoagulant platelets which may lead to the extensive thrombosis and thrombocytopenia observed in VITT patients.¹³

A role for complement has been proposed in VITT. Heat treatment of sera, which inactivates complement (56°C, 45 minutes), blocked aggregation in three out of seven patients (Figure 1A), while minor effects on aggregation were observed with compstatin (C3a inhibitor) and FUT-175 (C3, C4 and C5 inhibitor) (Figure 1B). These findings indicate that while complement is not critical, it may reinforce platelet activation. Eculizumab (anti-C5 monoclonal antibody) treatment has been reported in two patients with VITT where 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 VITT patients have been reported.²

We tested a variety of clinically used anti-platelet drugs and inhibitors of kinases downstream of FcγRIIA to determine if they could prevent platelet aggregation to patient sera.¹⁷ The COX inhibitor indomethacin, which works via same mechanism as aspirin, and the P2Y₁₂ inhibitor ticagrelor prevented aggregation to patient serum, as did the Src inhibitor dasatinib, and the Btk inhibitors ibrutinib and rilzabrutinib, with a significant reduction observed to the Syk inhibitor entospletinib (Figure 1C). This inhibition was irrespective of heterogeneity in VITT patient samples. All inhibitors were used at a concentration which fully inhibited aggregation to 3 µg/mL collagen (results not shown).

While these antiplatelet and kinase inhibitors are able to 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 however be limited 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 aspirin¹⁹ 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 thrombocytopenic patients cannot be recommended.²⁰⁻²² Rilzabrutinib, currently in trials for immune thrombocytopenia (ITP) with no bleeding or thrombotic events reported,²³ appears a more promising treatment to undergo 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 here 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 thrombocytopenic patients.²⁵ If ongoing treatment is required due to inadequate response to the scarce and expensive IVIg and plasma exchange, then these anti-platelet agents could potentially have a role, and warrant further evaluation.

Limitations of this study are the small sample size, and the differing treatments received prior to patient sample collection. Additionally, only a limited number of conditions could be tested due the volume of sera available, and aggregation was only measured over 10 minutes, with consensus now to examine aggregation to VITT patient serum over 30 minutes.

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

Acknowledgements

This work was supported by an Accelerator Grant (AA/18/2/34218) from the British Heart Foundation (BHF). CK is supported by the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie Actions Individual Fellowship grant agreement [No 893262], project PAELLA. SPW holds a

BHF Chair (CH03/003). The authors would like to thank Dr Mav Manji (University Hospitals Birmingham NHS Foundation Trust) for help with patient recruitment, Charlotte Stoneley and Matt Roberts (University Hospitals Birmingham NHS Foundation Trust) for help sourcing patient blood samples and information on the Immucor assay. We would like to thank OSP and SSP for sharing energy, Professor Adam Cunningham for insightful discussions, and Professor Alex Richter, Dr Adrian Shields and Dr Sian Faustini from the COCO study for AZD1222 vaccinated healthy donor samples, and Principia Biopharma for rilzabrutinib.

Authorship

CWS and PLRN designed and performed experiments, analysed data and wrote and revised the manuscript. SJM performed experiments and revised the manuscript. CK designed and performed experiments and revised the manuscript. YD generated reagents and revised the manuscript. SPW revised the manuscript and designed experiments. GCL and WAL recruited patients, revised the manuscript and contributed intellectually.

Declaration of interests

PLRN and SPW have received research grants from Novartis, Principia and Rigel Pharmaceuticals. PLRN has had honoraria from Bayer.

References

1. Greinacher A, Thiele T, Warkentin TE, et al. Thrombotic Thrombocytopenia after ChAdOx1 nCov-19 Vaccination. *N. Engl. J. Med.* 2021;
2. Schultz NH, Sørvoll IH, Michelsen AE, et al. Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. *N. Engl. J. Med.* 2021;
3. Greinacher A, Selleng K, Warkentin TE. Autoimmune heparin-induced thrombocytopenia. *J. Thromb. Haemost.* 2017;15(11):2099–2114.
4. Pottegård A, Lund LC, Karlstad Ø, et al. Arterial events, venous thromboembolism, thrombocytopenia, and bleeding after vaccination with Oxford-AstraZeneca ChAdOx1-S in Denmark and Norway: Population based cohort study. *BMJ.* 2021;373:1–10.
5. MHRA - Medicines & Healthcare products Regulatory Agency. Coronavirus vaccine - weekly summary of Yellow Card reporting. 2021;1–14.
6. British Society for Haematology. Guidance produced from the Expert Haematology Panel (EHP) focussed on Covid-19 Vaccine induced Thrombosis and Thrombocytopenia (VITT). 2021;
7. Warkentin TE, Hayward CPM, Smith CA, Kelly PM, Kelton JG. Determinants of donor platelet variability when testing for heparin-induced thrombocytopenia. *J. Lab. Clin. Med.* 1992;120(3):371–379.
8. Thiele T, Ulm L, Holtfreter S, et al. Frequency of positive anti-PF4/polyanion antibody tests after COVID-19 vaccination with ChAdOx1 nCoV-19 and BNT162b2. *Blood.* 2021;
9. Li J, Van Der Wal DE, Zhu G, et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. *Nat. Commun.* 2015;6:.
10. Rubino JG, Arnold DM, Warkentin TE, et al. A comparative study of platelet factor 4-enhanced platelet activation assays for the diagnosis of heparin-induced thrombocytopenia. *J. Thromb. Haemost.* 2021;19(4):1096–1102.
11. Vayne C, Guery E-A, Kizlik-Masson C, et al. Beneficial effect of exogenous platelet factor 4 for detecting pathogenic heparin-induced thrombocytopenia antibodies. *Br. J. Haematol.* 2017;179(5):811–819.
12. Nazy I, Jevtic SD, Moore JC, et al. Platelet-activating immune complexes identified in critically ill COVID-19 patients suspected of heparin-induced thrombocytopenia. *J. Thromb. Haemost.* 2021;(February):1–6.
13. Althaus K, Möller P, Uzun G, et al. Antibody-mediated procoagulant platelets in SARS-CoV-2- vaccination associated immune thrombotic thrombocytopenia. *Haematologica.* 2021;
14. Tiede A, Sachs UJ, Czwalinna A, et al. Prothrombotic immune thrombocytopenia after COVID-19 vaccine. *Blood.* 2021;
15. Althaus K, Marini I, Zlamal J, et al. Antibody-induced procoagulant platelets in severe COVID-19 infection. *Blood.* 2021;137(8):1061–1071.

16. Mastellos DC, Skendros P, Lambris JD. Is complement the culprit behind COVID-19 vaccine-related adverse reactions? *J. Clin. Invest.* 2021;131(11):1–5.
17. Arman M, Krauel K. Human platelet IgG Fc receptor FcγRIIA in immunity and thrombosis. *J. Thromb. Haemost.* 2015;13(6):893–908.
18. Zheng SL, Roddick AJ. Association of Aspirin Use for Primary Prevention With Cardiovascular Events and Bleeding Events: A Systematic Review and Meta-analysis. *JAMA.* 2019;321(3):277–287.
19. Bourguignon A, Arnold DM, Warkentin TE, et al. Adjunct Immune Globulin for Vaccine-Induced Thrombotic Thrombocytopenia. *N. Engl. J. Med.* 2021;1–9.
20. Becker RC, Bassand JP, Budaj A, et al. Bleeding complications with the P2Y12 receptor antagonists clopidogrel and ticagrelor in the PLATElet inhibition and patient Outcomes (PLATO) trial. *Eur. Heart J.* 2011;32(23):2933–2944.
21. Shatzel JJ, Olson SR, Tao DL, et al. Ibrutinib-associated bleeding: pathogenesis, management and risk reduction strategies. *J. Thromb. Haemost.* 2017;15(5):835–847.
22. Quintás-Cardama A, Kantarjian H, Ravandi F, et al. Bleeding diathesis in patients with chronic myelogenous leukemia receiving dasatinib therapy. *Cancer.* 2009;115(11):2482–2490.
23. Kuter DJ, Boccia R V, Lee E-J, et al. Phase I/II, Open-Label, Adaptive Study of Oral Bruton Tyrosine Kinase Inhibitor PRN1008 in Patients with Relapsed/Refractory Primary or Secondary Immune Thrombocytopenia. *Blood.* 2019;134(Supplement_1):87.
24. Cooper N, Altomare I, Thomas MR, et al. Assessment of thrombotic risk during long-term treatment of immune thrombocytopenia with fostamatinib. *Ther. Adv. Hematol.* 2021;12:1–12.
25. Awan FT, Thirman MJ, Patel-Donnelly D, et al. Entospletinib monotherapy in patients with relapsed or refractory chronic lymphocytic leukemia previously treated with B-cell receptor inhibitors: results of a phase 2 study. *Leuk. Lymphoma.* 2019;60(8):1972–1977.

Table 1. Summary of Clinical Characteristics of Patients with Vaccine-Induced Immune Thrombocytopenia and Thrombosis (VITT).

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	
Age	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	62342	6574	22903	31301	30324	6807	27000	
Fibrinogen at presentation (g/L) normal range 1.5 - 4	1.2	<0.35	0.98	1.1	1.07	<0.35	2.36	
Prothrombin Time Ratio at presentation normal range 0.8 – 1.2	1.2	1.5	1.3	1.2	1.1	1.4	1.1	
Activated Partial Thromboplastin Time 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 range \leq 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 range \leq 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 range ≤ 8%	0.64%	3.68%	1.43%	N/A (not done)	N/A (not done)	N/A (not done)	4.38%
Clinical	CVST	CVST	Ischaemic stroke	CVST	CVST	CVST	CVST	CVST
Number of days post vaccine at presentation	14	12	10	14	11	9	12	
Presentation symptoms	Headaches; Haematuria; Petechial rash; Subsequent development of left sided weakness	Occipital headache	Headache for 2-3 days; Collapse; Expressive dysphasia	Headache	Headache; Aura; Petechial rash	Headache and vomiting for few hours; Followed by reduced conscious level	Headache for 1 week; Development of right sided weakness; Subsequent seizure and collapse	
Co-morbidities	Prostatitis	None	None	Hypothyroidism ; Fibromyalgia; Anxiety	None	None	None	None
Medications	None	None	None	Levothyroxine; Sertraline; Amitriptyline	None	None	None	None
Imaging findings at presentation	CVST; Subarachnoid haemorrhage	CVST; Subarachnoid haemorrhage; Intraparenchymal haemorrhage	Acute left ICA thrombus with multiple left middle MCA territory infarctions	CVST; Intraparenchymal haemorrhage	CVST	CVST; Left sided intracerebral haemorrhage; Midline shift	CVST; Subarachnoid and intraparenchymal haemorrhage; globalised brain atrophy	

Immunosuppression regime used	IVIg 0.5 g/kg OD for 2 consecutive days Dexamethasone 20mg OD for 3 days	IVIg 1 g/kg for 2 consecutive days Dexamethasone 40 mg OD for 4 days	IVIg 1 g/kg single dose Dexamethasone 40 mg OD for 4 days	IVIg 1 g/kg single dose Dexamethasone 40 mg OD for 4 days	IVIg 1 g/kg on 2 non-consecutive days Dexamethasone 40 mg OD for 3 days	IVIg 1 g/kg on 2 non-consecutive days Dexamethasone 40 mg OD for 4 days	IVIg 1 g/kg single dose Dexamethasone 40 mg (two doses)
Anticoagulant / Antiplatelet regime used	Argatroban Fondaparinux 7.5mg SC OD (when platelets normalised)	Argatroban	Fondaparinux 7.5mg OD Apixaban 5mg BD (on discharge)	Fondaparinux 2.5mg SC OD (whilst platelets <50 x10 ⁹ /L) Fondaparinux 7.5mg SC OD (when platelets ≥50 x10 ⁹ /L) Dabigatran 150mg BD (on discharge)	Fondaparinux 7.5 mg SC OD	Argatroban Fondaparinux 7.5mg SC OD (when platelets ≥50 x10 ⁹ /L)	None
Other treatments required	Intubation	Intubation, Thrombectomy	Thrombectomy	None	Plasma exchange	Intubation; Thrombectomy; Decompressive ; Craniotomy; Plasma exchange; Platelet transfusion	Intubation; mannitol
Timing of first serum sample	Post-IVIg and Dexamethasone	Post-single dose of Dexamethasone	Pre-treatment	Pre-treatment	Post-IVIg and Dexamethasone	Post-IVIg and Dexamethasone	Pre-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 post IVIg that platelet count rose	N/A – Nadir 59	2 days	N/A – nadir 52	3 days	4 days	1 day	N/A - died <24

>50 x10⁹/L	x10 ⁹ /L (platelets 100 x10 ⁹ /L two days after first IVIg infusion)		x10 ⁹ /L (Platelets 198 x10 ⁹ /L three days after IVIg infusion)				hours after IVIg
Outcome	Clinically recovered at time of discharge from hospital after a 26 day admission	Death (support withdrawn following confirmation of brainstem death)	Clinically recovered at time of discharge from hospital after a 10 day admission Ongoing mild right hand weakness and expressive dysphasia	Clinically recovered at time of discharge from hospital after a 16 day admission 2x further admissions with headaches and drops in platelet counts. No further CVST. Treated with (1 st admission) Prednisolone then (2 nd admission) IVIg and Rituximab. Remains in hospital	Clinically recovered at time of discharge from hospital after a 12 day admission	36 day intensive care admission. Ventilator associated pneumonia. Limited Neurological Recovery. Remains in hospital	Death 24 hours after admission (rapid development of global ischaemia before thrombectomy could be performed)

BD, twice daily; CPAP, continuous positive airway pressure; CVST, cerebral venous sinus thrombosis; ICA, internal carotid artery; ICH, intracerebral haemorrhage; IVIg, intravenous immunoglobulin; MCA, middle cerebral artery; N/A, not available; OD, once daily; SC, subcutaneous; PEX, plasma exchange.

Figure Legends

Figure 1. Serum from patients with VITT induces platelet aggregation via the FcγRIIA, and can be blocked by inhibition of cyclooxygenase, P2Y₁₂, Src and Btk. Washed platelets (2×10^8 /mL) were stimulated with serum (15:1, v/v) and aggregation measured by light transmission aggregometry. **(Ai)** Representative aggregation traces for AZD1222 vaccinated healthy donors (HD) or patients with VITT (P) serum pre- and post-IVIg treatment in the presence of Tyrode's buffer, 10 μg/mL IV.3 F(ab), low concentration heparin (0.2 U/ml) or following heat inactivation of complement (56°C, 45 minutes) and post-plasma exchange. Quantification of area under the curve (AUC) for 10 minutes for **(Aii)** P2, P3, P4, P7 pre- and post-IVIg samples and **(Aiii)** P1, P5 and P6 post-IVIg and plasma exchange samples. Mean ± SEM, n=3. Statistical analysis was by two-way ANOVA with Dunnett multiple comparisons (vs Serum **(Aii)**; vs Post-IVIg serum **(Aiii)**), *p<0.05, ns: non-significant. **(B)** The effect of complement inhibitors compstatin (28 μM), FUT-175 (10 μM) or vehicle on aggregation to VITT 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 P4, P5 and P7 respectively). Statistical analysis was by one-way ANOVA with Dunnett 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 to VITT 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, 1 repeat P5 and P7 respectively). Statistical analysis was by one-way ANOVA with Dunnett multiple comparisons, *p<0.05, ns: non-significant.

Anti-Platelet Drugs Block Platelet Activation by Vaccine-Induced Immune Thrombocytopenia and Thrombosis (VITT) Patient Serum

Christopher W. Smith,¹ Samantha J. Montague¹, Caroline Kardeby,¹ Ying Di,¹ Gillian C. Lowe,² William A. Lester,² Steve P. Watson,¹ Phillip L.R. Nicolson,^{1,2}

¹ Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK

² Comprehensive Care Haemophilia Centre, University Hospitals Birmingham NHS Foundation Trust, Birmingham, B15 2TH, UK

Corresponding author: Phillip L.R. Nicolson, email: p.nicolson@bham.ac.uk

Supplemental Information

Methods

Patients and Ethical approval

Patients presenting with thrombosis and thrombocytopenia, occurring after AZD1222 vaccination were recruited. Ethical approval for collecting blood from patients was approved under research ethics 15/NW/0079, and from healthy volunteers by Birmingham University Internal Ethical Review (ERN_11-0175). Samples from AZD1222 vaccinated individuals who did not develop VITT were collected as part of the COCO study, approved by the London - Camden and Kings Cross Research Ethics Committee (reference 20/HRA/1817).

Antibodies and reagents

Mouse monoclonal IgG2b antibody against human CD32 (IV.3) was purified from hybridoma cells supernatant, and IV.3 F(ab) fragment made using Pierce Fab Preparation kit (Thermo Fisher Scientific). Eptifibatide was from GSK. Ibrutinib, R406, and entospletinib were from Selleckchem. Rilzabrutinib was provided by Principia BioPharma. All other reagents were from Sigma-Aldrich.

Serum preparation

Serum was collected following centrifugation (2000×g, 10 minutes, room temperature [RT]) of clotted whole blood. Patient sera was collected before and after treatment with dexamethasone, IVIg and plasma exchange (see Table 1).

Human platelet preparation

Acid citrate dextrose (1:10, v/v) was added citrated blood taken from healthy, drug-free volunteers and centrifuged (200×g, 20 minutes, RT). Platelet rich plasma isolated and centrifuged with 0.2µg/mL prostacyclin (1000×g, 10 minutes, RT). Platelets were washed again in modified-Tyrode's-HEPES buffer and prostacyclin, before resuspension in modified-Tyrode's-HEPES and rested before testing.

Light transmission aggregometry (LTA)

Aggregation was measured using a light transmission aggregometer (Model 700, ChronoLog) for 10 minutes, 1200 rpm, 37°C following washed platelet stimulation with serum (15:1, v/v). IV.3 F(ab) and inhibitor pre-incubation was for 5 and 10 minutes respectively. Aggregations were conducted in washed platelets from 4 healthy donors

known to respond.

Statistical analysis

All data presented as mean \pm SEM, $p < 0.05$ was considered statistically significant. Statistical analysis was performed in GraphPad Prism 9 using one or two-way ANOVA with Dunnett corrections for multiple comparisons.

Figure 1

