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Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a rare adverse effect of COVID-19 adenoviral vector vaccines[1–3]. VITT resembles heparin-induced thrombocytopenia (HIT) as it is associated with platelet-activating antibodies against platelet factor 4 (PF4)[4]; however, patients with VITT develop thrombocytopenia and thrombosis without heparin exposure. The objective of this study was to determine the binding site on PF4 of antibodies from patients with VITT. Using alanine scanning mutagenesis[5], we determined the binding of VITT anti-PF4 antibodies (n=5) was restricted to 8 surface amino acids, all of which were located within the heparin binding site on PF4, and the binding was inhibited by heparin. In contrast, HIT sampled (n=10) bound to amino acids corresponding to 2 different sites on PF4. Using biolayer interferometry, we demonstrated VITT anti-PF4 antibodies had a stronger binding response against PF4 and PF4/heparin complexes than HIT antibodies; albeit, with similar dissociation rates. Our data indicates VITT antibodies can mimic the effect of heparin by binding to a similar site on PF4, allowing PF4 tetramers to cluster and form immune complexes, which in turn cause FcγRIIa-dependent platelet activation. These results provide an explanation for VITT antibody-induced platelet activation that could contribute to thrombosis.

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a rare, but serious adverse effect of adenoviral vector vaccines against SARS-CoV-2 virus. The clinical picture of VITT is moderate to severe thrombocytopenia plus arterial and/or venous thrombi, often occurring in unusual locations[1–3]. These findings resemble the immunological drug-reaction heparin-induced thrombocytopenia (HIT), which presents clinically as thrombocytopenia and thrombosis in patients previously exposed to heparin[4]. VITT most closely resembles the exceptionally rare spontaneous HIT, which occurs in the absence of heparin[6,7].

HIT is caused by immunoglobulin G (IgG) antibodies that bind to neoepitopes on platelet factor 4 (PF4/CXCL4), a 70 amino acid cationic protein contained within platelets[8,9]. The neoepitopes become exposed after heparin, a large anionic polysaccharide, binds to a specific site on PF4, causing PF4 tetramers to cluster together. The IgG-specific antibodies bind to PF4/heparin forming immune complexes, which activate platelets through FcγRIIa receptors causing intense platelet activation and release of procoagulant rich microparticles[10]. Other cells, including monocytes, are also activated by these immune complexes, which amplifies the hypercoagulable state in HIT patients[11]. It has been postulated that VITT has a similar pathophysiology to HIT, and several investigators have demonstrated the presence of high levels of anti-PF4 antibodies in VITT samples[1–3,12]. However, VITT is a unique syndrome since it occurs without heparin exposure, and the pattern of platelet reactivity, *in vitro*, does not demonstrate typical heparin-dependence, as seen with HIT.

In this report, we described the binding site and characteristics of anti-PF4 antibodies in VITT patients that developed in response to COVID-19 adenoviral vector vaccination. We found VITT patients had anti-PF4 antibodies that bound to a highly restricted site on PF4 corresponding to the heparin binding site. These antibodies can form platelet-activating immune complexes without heparin, potentially causing thrombocytopenia and clotting observed in VITT.

Patient demographics

VITT samples (n = 5) were referred to the McMaster Platelet Immunology Laboratory for confirmatory diagnosis. All VITT patients had received a single dose of the ChAdOx1 nCoV-19 vaccine (AstraZeneca COVID-19 Vaccine, AstraZeneca; COVIDSHIELD, Verity Pharmaceuticals and Serum Institute of India) and subsequently developed thrombocytopenia and thrombosis; mean age was 44 years (range: 35 – 72 years) and 2/5 (40%) were female. The time from first dose of the ChAdOx1 nCoV-19 vaccine to sample collection was 14 to 40 days (mean: 28 days). All VITT samples had antibodies against PF4 [mean optical density (OD): 2.71, range: 0.763 – 3.347).

VITT samples were compared to HIT patients (n = 10) who had thrombocytopenia after receiving heparin and had a high clinical probability score (4Ts score ≥ 4) with detectable anti-PF4/heparin antibodies and evidence of platelet activation *in vitro*. HIT patients had a mean age of 69 years (range: 52 – 81 years) and 5/10 (50%) were female. Nine of 10 (90%) HIT patients experienced thrombosis. The time from heparin initiation to sample collection was 6 – 27 days (mean: 14.3 days). All HIT samples had detectable anti-PF4/heparin antibodies (mean OD: 3.10, range: 2.329 – 3.897).

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Platelet activation profiles of VITT antibodies

Using a functional platelet activation assay [serotonin release assay (SRA)], we showed VITT and HIT patient samples had unique patterns of platelet reactivity *in vitro*. VITT samples did not demonstrate any heparin-dependence on platelet activation *in vitro* (Extended Data Figure 1A). All VITT samples demonstrated strong, dose-dependent PF4-mediated platelet activation with 50 μg/mL of PF4 (Extended Data Figure 1B). Complete inhibition of platelet activation by VITT and HIT samples was achieved with the addition of the FcγRIIa-blocking monoclonal antibody IV.3[4]. These results indicate that VITT antibodies require PF4 for platelet activation, which is mediated by engagement of FcγRIIa receptors consistent with the effects of an immune complex. In contrast, HIT samples activated platelets with two distinct profiles. HIT patients with heparin-dependent (HD) antibodies were patients who were exposed to heparin and their antibodies activated platelets in the SRA in the presence of heparin (Extended Data Figure 1C). HIT patients with heparin-independent (HI) antibodies were patients who were exposed to heparin and their antibodies activated platelets in the SRA with and without the addition of heparin. (Extended Data Figure 1C). All HIT samples also activated platelets with the addition of PF4 (Extended Data Figure 1D).

Binding Site of VITT Antibodies on PF4

Previous studies have shown that the binding epitopes of HIT antibodies are non-contiguous and conformation-dependent[13]. Therefore, to identify the specific amino acid targets of VITT antibodies on PF4, we used alanine scanning mutagenesis and produced 70 unique recombinant PF4 mutants, each differing by a single amino acid[5]. We defined a critical binding amino acid as a corresponding PF4 mutant that caused a greater than 50% reduction in binding compared to wild-type PF4. We identified 8 surface amino acids that were necessary for binding of VITT samples (R22, H23, E28, K46, N47, K50, K62, and K66; Table 1 and Figure 1a, red colour). The PF4 mutants R22A and E28A affected the binding for all five VITT samples. This restricted epitope is consistent with limited B-cell clonality and suggests that the binding site of VITT antibodies is to a specific site on PF4. We observed that 4 of the 8 amino acids that comprised the VITT epitope (R22, H23, K46, and K66) corresponded to amino acids on PF4 to which heparin binds (Figure 1b, brown colour)[14]. We postulate binding of VITT anti-PF4 antibodies to the heparin binding site on the PF4 tetramer explains why the addition of heparin *in vitro* does not augment platelet activation, as seen in HIT; rather it inhibits platelet activation, presumably by displacing VITT anti-PF4 antibodies. Furthermore, in a PF4 enzyme immunoassay (EIA), we showed VITT antibody binding to PF4 were inhibited by therapeutic concentrations of heparin in four of the available VITT samples (Extended Data Figure 2). Our results explain why some VITT samples tested in studies by Schultz *et al*[2] and Greinacher *et al*[1] were inhibited by therapeutic doses of heparin. Our findings suggest VITT antibodies cause platelet activation through a similar heparin-like mechanism by stabilizing complexes of PF4, aligning the Fc-portion in close proximity and crosslinking FcγRIIa receptors on platelets, similar to that of the monoclonal antibody, 1E12, which can activate platelets independent of heparin[15].

The VITT binding site on PF4 was then compared with that of HIT. When all the critical amino acids from screening the 10 HIT patients were combined, there was a total of 10 amino acids (L8, C10, C12, T16, R22, Q40, N47, C52, L53, D54, K61, K66, L67; Table 1) that were part of the HIT epitopes, in different combinations. No single common amino acid was critical for binding of all 10 HIT samples, likely due to the polyclonal nature of the antibodies[5]. PF4 mutants L8A, C10A, C12A, T16A, C52A, D54A, and K61A represented the most common amino acids to affect the binding of HIT antibodies and were common among 6 of the HIT samples. When displayed on the PF4 tetramer, we identified one site on PF4 that all HIT (10/10) samples targeted (Figure 1c and 1d, blue colour). In addition, 6 of the 10 HIT samples targeted an additional site (Figure 1d, red colour) similar to the VITT site that was within the heparin binding site. Unlike VITT samples, none of the HIT samples were restricted to the heparin binding site. This is consistent with previous observations that some HIT samples contain two types of platelet-activating anti-PF4/heparin antibodies[16]. The HIT antibodies bound to a similar site as KKO, a monoclonal antibody against PF4/heparin complexes, thus providing an explanation as to why HIT antibodies, but not VITT antibodies, require heparin to crosslink PF4 tetramers[5].

In addition to clarifying the binding site of VITT antibodies, these results provide an explanation for why some rapid HIT immunoassays may yield false-negative results for VITT[17]. One rapid HIT immunoassay, the latex immunoturbidimetric assay [HemosIL® HIT-Ab$_{(PF4-H)}$] uses KKO to aggregate complexes of PF4/heparin. Since all HIT samples have antibodies that bind to the same site as KKO, the HIT samples compete with KKO in binding the PF4/heparin complexes. In contrast, VITT antibodies bind to a different site on PF4 than KKO, and thus do not compete for binding.

Binding kinetics of VITT antibodies

The binding response of VITT samples (n = 5), HIT samples (n = 10) and healthy control samples (n = 10) to PF4 and PF4/heparin were tested using bio-layer interferometry (BLI). Binding responses are a measure of antigen-specific antibodies present in a given sample. When healthy control samples (n = 10) were tested with immobilized PF4 and PF4/heparin, the mean binding response [nm shift ± 2 standard deviations (SD)] was 0.0059 ± 0.11 nm and 0.031 ± 0.11 nm, respectively. All VITT (n = 5) and HIT (n = 10) samples were above the mean + 2SD cut-off of 0.12 nm with immobilized PF4 and 0.14 nm with immobilized PF4/heparin, indicating a positive binding result. The mean binding response (nm shift ± SD) was 1.82 ± 0.88 nm for VITT patients; and 0.82 ± 0.72 nm for HIT patients ($p < 0.05$) with immobilized PF4. Similarly, the mean binding response was 1.24 ± 0.70 nm for VITT samples and 0.62 ± 0.45 nm for HIT patients ($p < 0.05$) with PF4/heparin complexes. Therefore, the binding response in VITT sera was significantly higher than HIT sera and healthy controls with both PF4 and PF4/heparin, indicating a stronger antibody response in VITT patients.

We further compared the binding response for the VITT samples to the two groups of HIT samples, with HD (n = 4) and HI (n = 6) antibodies. The mean binding response for HIT patients with HD antibodies was 0.29 ± 0.18 nm and for HIT patients with HI antibodies was 1.18 ± 0.73 nm against immobilized PF4 (Figure 2c). When samples were tested against immobilized PF4/heparin complexes, the mean binding response was 0.30 ± 0.09 nm for HIT patients with HD antibodies and 0.83 ± 0.48 nm for HIT with HI antibodies patients (Figure 2d). The binding response of VITT samples was significantly higher than HIT patients with HD antibodies and healthy controls with both PF4 and PF4/heparin (VITT vs. HIT with HD antibodies: $p < 0.01$, VITT vs. healthy controls: $p < 0.001$). In contrast, binding response from VITT samples was similar to HIT patients with HI antibodies. VITT samples also had a significantly higher binding response to PF4 when compared to PF4/heparin ($p < 0.05$), consistent with an inhibitory effect of heparin on the binding of VITT antibodies to PF4 (Extended Data Figure 2).

To confirm the binding responses in samples were due to anti-PF4 antibodies and not due to other serum factors, total IgG was purified from two VITT samples and then tested for their ability to bind PF4 and PF4/heparin. However, due to sample constraints, further purification of specific VITT anti-PF4 antibodies were not performed which presents a potential limitation for this study. The binding response of total IgG isolated from both VITT samples against PF4 and PF4/heparin were similar to the binding responses observed with their respective sera,

indicating that the binding responses are due to the anti-PF4 antibodies (Extended Data Figure 3). In addition, two VITT, one HIT, and two healthy control samples were re-tested in separate experiments and demonstrated reproducibility (Extended Data Figure 4).

With polyclonal antibodies interacting with an antigen, the dissociation rate, which is concentration-independent, can be measured. The mean dissociation rate (k_{off} s^{-1} ± SD) was $6.32 \times 10^{-3} \pm 0.0077$ s^{-1} for VITT samples; $2.34 \times 10^{-3} \pm 0.0041$ s^{-1} for HIT patients with HD antibodies; and $9.65 \times 10^{-4} \pm 0.0016$ s^{-1} for HIT patients with HI antibodies with immobilized PF4 (Figure 2e). Similarly, the mean dissociation rate (k_{off} s^{-1} ± SD) was $3.47 \times 10^{-3} \pm 0.0029$ s^{-1} for VITT patients; $4.56 \times 10^{-4} \pm 0.0002$ s^{-1} for HIT patients with HD antibodies; and $1.07 \times 10^{-3} \pm 0.0018$ s^{-1} for HIT patients with HI antibodies with immobilized PF4/heparin (Figure 2f). There was no statistically significant difference in the dissociation rates of the VITT and the two HIT groups with both PF4 and PF4/heparin (PF4: VITT vs. HIT patients with HD antibodies $p = 0.482$, VITT vs. HIT patients with HI antibodies $p = 0.220$; and for PF4/heparin, VITT vs. HIT patients with HD antibodies $p = 0.104$; VITT vs. HIT patients with HI antibodies $p = 0.160$). The low dissociation rates likely allow for sufficient binding and formation of immune complexes to induce platelet activation by crosslinking FcγRIIa receptors on platelets. As human samples are polyclonal, affinity could not be determined, however, binding responses and dissociation rates demonstrate the strength of the immune response and the avidity of the polyclonal samples, respectively.

Monoclonal antibodies against PF4, such as KKO[18] and 1E12[15] facilitate the formation of ultra-large complexes of PF4 on the platelet surface. Previous studies using antibodies from HIT patients[19] and monoclonal antibodies that resemble HIT antibodies[15] have demonstrated that the higher affinity of HI antibodies in some HIT patients can cluster PF4 tetramers and create platelet-activating immune complexes, in the absence of heparin. Similarly, we showed that anti-PF4 antibodies from VITT patients have comparable binding response and avidity to the antibodies from HIT patients, especially HIT patients with HI antibodies, implying they can cluster PF4 tetramers and create the same ultra-large platelet-activating immune complexes (Extended Data Figure 5).

It has been observed that 50% of HIT samples tested in the SRA, cause platelet activation *in vitro* without the addition of heparin[20–22]. As such, the HIT samples were separated into 2 groups, those HD antibodies and those with HI antibodies. The distinguishing factor that defines anti-PF4 antibodies from VITT patients is that these patients, unlike HIT patients, have not been exposed to heparin and their anti-PF4 antibodies are restricted to the heparin binding site.

In this report, we demonstrated anti-PF4 antibodies in VITT patients can induce platelet activation via FcγRIIa receptors in the presence of PF4, without heparin. However, other serum factors could also contribute to platelet activation. Previous studies have demonstrated antibodies from VITT patients were able to activate platelets and cause platelet aggregation in the presence of adenoviral particles in a dose-dependent manner[1,23,24]. Thus, it is possible platelet activation caused by anti-PF4 antibodies in VITT patients may not be the only factor leading to the development of thrombotic events. HIT is also propagated by various pro-thrombotic mechanisms that could also be important in VITT, including Fc-receptor polymorphisms[25], monocyte-activation and tissue factor production[26], and procoagulant microparticle generation[10].

This study offers an explanation for VITT-mediated platelet activation. VITT patients in our study demonstrated similar antibody characteristics that bound PF4 at the same site as heparin. VITT antibodies form immune complexes without the addition of heparin or other co-factors, activate platelets and potentially other cells via FcγRIIa receptors which in turn, could initiate coagulation at multiple points causing thrombocytopenia and thrombosis.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-03744-4.

1. Greinacher, A. *et al*. Thrombotic Thrombocytopenia after ChAdOx1 nCov-19 Vaccination. *N Engl J Med*, https://doi.org/10.1056/NEJMoa2104840 (2021).
2. Schultz, N. H. *et al*. Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. *N Engl J Med*, https://doi.org/10.1056/NEJMoa2104882 (2021).
3. Scully, M. *et al*. Pathologic Antibodies to Platelet Factor 4 after ChAdOx1 nCoV-19 Vaccination. *N Engl J Med*, https://doi.org/10.1056/NEJMoa2105385 (2021).
4. Kelton, J. G. *et al*. Heparin-induced thrombocytopenia: laboratory studies. *Blood* **72**, 925-930 (1988).
5. Huynh, A. *et al*. Characterization of platelet factor 4 amino acids that bind pathogenic antibodies in heparin-induced thrombocytopenia. *J Thromb Haemost* **17**, 389-399, https://doi.org/10.1111/jth.14369 (2019).
6. Warkentin, T. E., Makris, M., Jay, R. M. & Kelton, J. G. A spontaneous prothrombotic disorder resembling heparin-induced thrombocytopenia. *Am J Med* **121**, 632-636, https://doi.org/10.1016/j.amjmed.2008.03.012 (2008).
7. Hwang, S. R. *et al*. Cerebral venous sinus thrombosis associated with spontaneous heparin-induced thrombocytopenia syndrome after total knee arthroplasty. *Platelets*, 1-5, https://doi.org/10.1080/09537104.2020.1828574 (2020).
8. Amiral, J. *et al*. Antibodies to macromolecular platelet factor 4-heparin complexes in heparin-induced thrombocytopenia: a study of 44 cases. *Thromb Haemost* **73**, 21-28 (1995).
9. Cai, Z. *et al*. Atomic description of the immune complex involved in heparin-induced thrombocytopenia. *Nat Commun* **6**, 8277, https://doi.org/10.1038/ncomms9277 (2015).
10. Warkentin, T. E. *et al*. Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: an explanation for the thrombotic complications of heparin-induced thrombocytopenia. *Blood* **84**, 3691-3699 (1994).
11. Rauova, L. *et al*. Monocyte-bound PF4 in the pathogenesis of heparin-induced thrombocytopenia. *Blood* **116**, 5021-5031, https://doi.org/10.1182/blood-2010-03-276964 (2010).
12. Thiele, T. *et al*. Frequency of positive anti-PF4/polyanion antibody tests after COVID-19 vaccination with ChAdOx1 nCoV-19 and BNT162b2. *Blood*, https://doi.org/10.1182/blood.2021012217 (2021).
13. Horsewood, P., Warkentin, T. E., Hayward, C. P. & Kelton, J. G. The epitope specificity of heparin-induced thrombocytopenia. *Br J Haematol* **95**, 161-167, https://doi.org/10.1046/j.1365-2141.1996.d01-1876.x (1996).
14. Mayo, K. H. *et al*. Heparin binding to platelet factor-4. An NMR and site-directed mutagenesis study: arginine residues are crucial for binding. *Biochem J* **312** (Pt 2), 357-365 (1995).
15. Vayne, C. *et al*. Characterization of New Monoclonal PF4-Specific Antibodies as Useful Tools for Studies on Typical and Autoimmune Heparin-Induced Thrombocytopenia. *Thromb Haemost* **121**, 322-331, https://doi.org/10.1055/s-0040-1717078 (2021).
16. Nguyen, T. H., Medvedev, N., Delcea, M. & Greinacher, A. Anti-platelet factor 4/polyanion antibodies mediate a new mechanism of autoimmunity. *Nat Commun* **8**, 14945, https://doi.org/10.1038/ncomms14945 (2017).
17. Vayne, C. *et al*. PF4 Immunoassays in Vaccine-Induced Thrombotic Thrombocytopenia. *N Engl J Med*, https://doi.org/10.1056/NEJMc2106383 (2021).
18. Sachais, B. S. *et al*. Dynamic antibody-binding properties in the pathogenesis of HIT. *Blood* **120**, 1137-1142, https://doi.org/10.1182/blood-2012-01-407262 (2012).
19. Bui, V. C. & Nguyen, T. H. The Role of Single-Molecule Force Spectroscopy in Unraveling Typical and Autoimmune Heparin-induced Thrombocytopenia. *Int J Mol Sci* **19**, https://doi.org/10.3390/ijms19041054 (2018).
20. Socher, I., Kroll, H., Jorks, S., Santoso, S. & Sachs, U. J. Heparin-independent activation of platelets by heparin-induced thrombocytopenia antibodies: a common occurrence. *J Thromb Haemost* **6**, 197-200, https://doi.org/10.1111/j.1538-7836.2007.02815.x (2008).
21. Warkentin, T. E., Arnold, D. M., Nazi, I. & Kelton, J. G. The platelet serotonin-release assay. *Am J Hematol* **90**, 564-572, https://doi.org/10.1002/ajh.24006 (2015).
22. Padmanabhan, A. *et al*. Heparin-independent, PF4-dependent binding of HIT antibodies to platelets: implications for HIT pathogenesis. *Blood* **125**, 155-161, https://doi.org/10.1182/blood-2014-06-580894 (2015).
23. Tiede, A. *et al*. Prothrombotic immune thrombocytopenia after COVID-19 vaccine. *Blood*, https://doi.org/10.1182/blood.2021011958 (2021).
24. Althaus, K. *et al*. Antibody-mediated procoagulant platelets in SARS-CoV-2- vaccination associated immune thrombotic thrombocytopenia. *Haematologica*, https://doi.org/10.3324/haematol.2021.279000 (2021).
25. Arepally, G., McKenzie, S. E., Jiang, X. M., Poncz, M. & Cines, D. B. Fc gamma RIIA H/R 131 polymorphism, subclass-specific IgG anti-heparin/platelet factor 4 antibodies and clinical course in patients with heparin-induced thrombocytopenia and thrombosis. *Blood* **89**, 370-375 (1997).
26. Kasthuri, R. S. *et al*. PF4/heparin-antibody complex induces monocyte tissue factor expression and release of tissue factor positive microparticles by activation of FcgammaRI. *Blood* **119**, 5285-5293, https://doi.org/10.1182/blood-2011-06-359430 (2012).

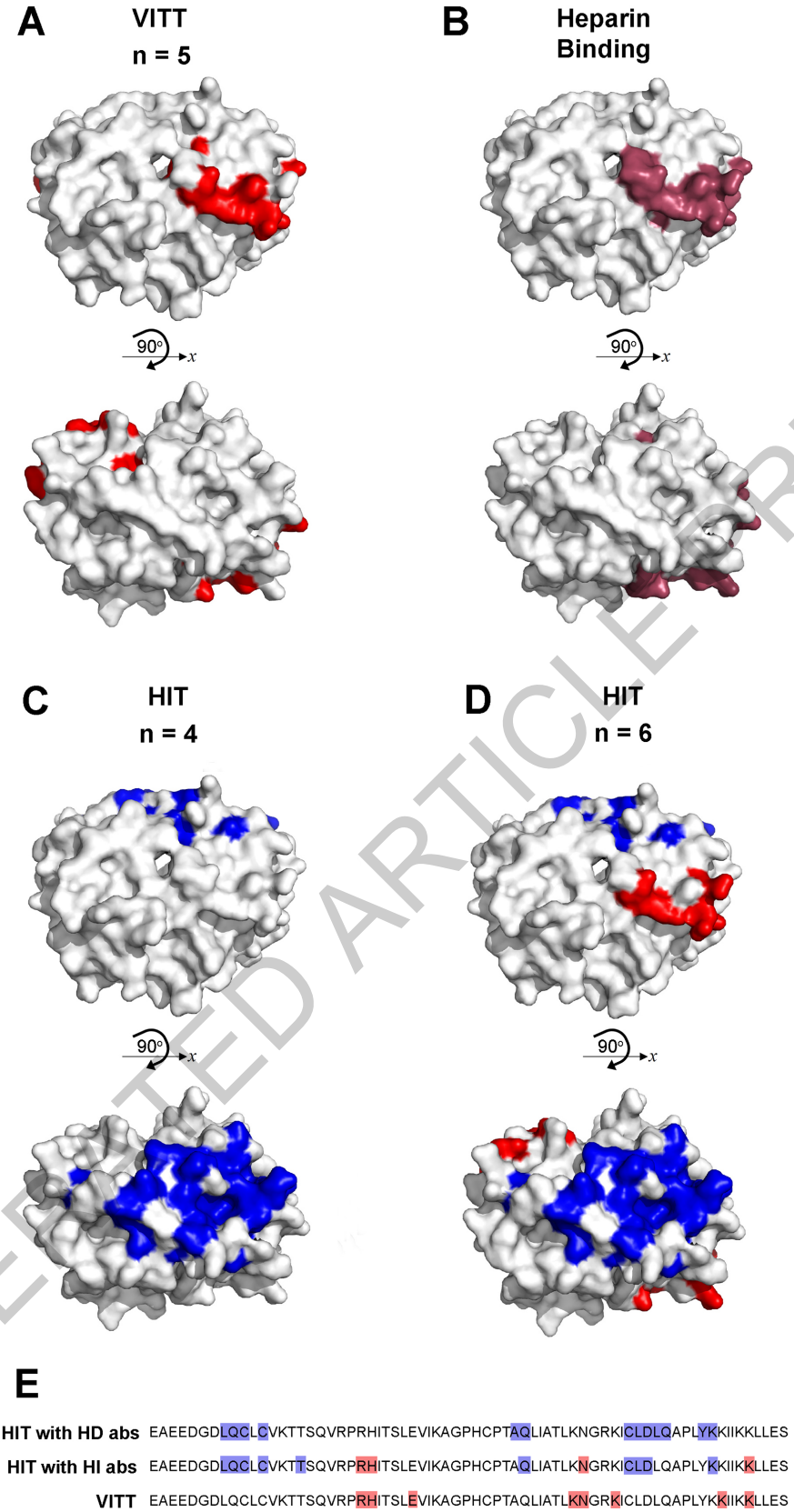

Fig. 1 | PF4 amino acids critical for binding antibodies using alanine scanning mutagenesis. A) VITT samples (n = 5) showed the binding site (coloured in red) aligns within **B)** the heparin binding site on PF4 (coloured in brown). **C)** For HIT samples (n = 10), one main binding site was identified for all samples as shown in blue and **D)** an additional binding site in 6/10 (60%) of the HIT samples, colored in red, which aligns within the heparin binding site on PF4. **E)** The amino acids that are predicted to be part of the binding sites for VITT and HD and HI antibodies in HIT are highlighted on the primary sequence of PF4. HD abs = Heparin-dependent antibodies; HI abs = Heparin-independent antibodies. Images are modified from PDB 1RHP.

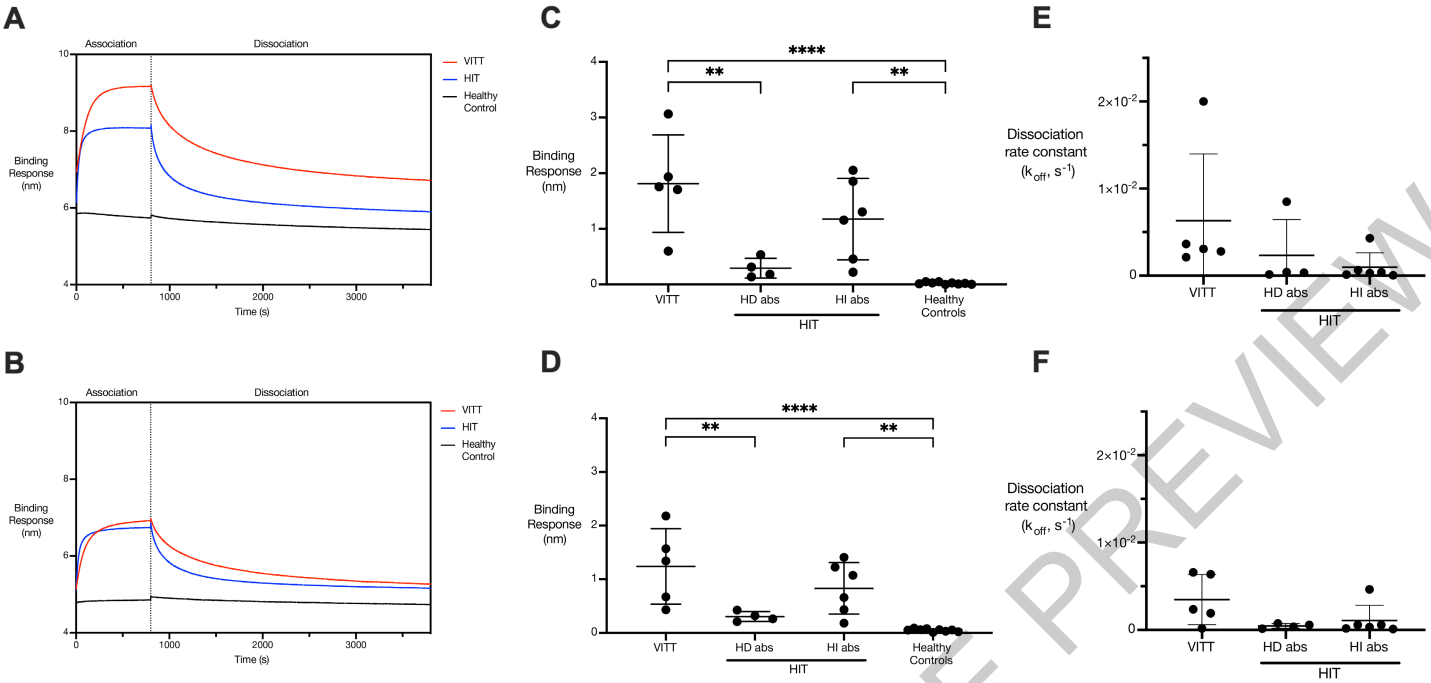

Fig. 2 | Association and dissociation of antibodies against PF4 and PF4/heparin using biolayer interferometry (BLI). Representative VITT, HIT, and healthy control antibody binding A) biotinylated PF4 and B) biotinylated PF4/heparin immobilized on streptavidin biosensors. Binding responses with C) PF4 and D) PF4/heparin and dissociation rates (k_{off}) with E) PF4 and F) PF4/heparin of samples from VITT patients (n = 5), HIT patients (n = 10), and healthy controls (n = 10) are also shown. HIT patients with HD antibodies (n = 4) are typical HIT patients and HIT patients with HI antibodies (n = 6) are HIT patients who have heparin-independent platelet activation in the SRA. Results show that VITT samples have higher binding responses to both PF4 and PF4/heparin than HIT patients with HD antibodies (PF4: p = 0.0017, PF4/heparin: p = 0.0090) and healthy control samples. HD abs = Heparin-dependent antibodies; HI abs = Heparin-independent antibodies. Values are shown as a mean binding response (nm) ± SD and mean dissociation rates k_{off} (s^{-1}) ± SD. Statistical analysis by two-tailed Mann-Whitney test, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0005.

Table 1 | PF4 amino acids identified by alanine scanning mutagenesis for binding VITT (n = 5) and HIT (n = 10) antibodies

Important PF4 amino acids for VITT antibodies			
PF4 Mutant	**Mean loss of binding compared to wild-type PF4 (%, n = 5)**	**Range (%, n = 5)**	**Number of VITT samples affected**
R22A	79.9	51.8 – 91.7	5/5
H23A	68.0	26.7 – 77.5	3/5
E28A	68.5	48.4 – 88.1	5/5
K46A	72.6	17.5 – 84.2	4/5
N47A	54.3	31.6 – 66.4	5/5
K50A	82.2	11.6 – 90.5	4/5
K62A	58.8	15.3 – 77.3	3/5
K66A	54.1	2.2 – 88.8	4/5
Important PF4 amino acids for HIT antibodies			
PF4 Mutant	**Mean loss of binding compared to wild-type PF4 (%, n = 10)**	**Range (%, n = 10)**	**Number of HIT samples affected**
L8A	48.6	12.4 – 76.0	6/10
C10A	49.4	5.0 – 73.3	6/10
C12A	45.7	4.8 – 74.7	7/10
T16A	41.1	4.0 – 78.4	6/10
R22A	36.2	1.3 – 63.0	5/10
Q40A	35.5	2.2 – 75.5	3/10
N47A	31.4	3.9 – 80.1	3/10
C52A	55.8	25.0 – 73.3	8/10
L53A	51.3	36.1 – 74.8	5/10
D54A	47.1	0.8 – 76.0	6/10
K61A	47.4	1.7 – 82.2	6/10
K66A	40.4	5.1 – 89.6	5/10
L67A	48.5	6.4 – 85.8	5/10

Methods

Study Participants

Participants included patients diagnosed with VITT (n = 5), patients diagnosed with HIT (n = 10), and healthy volunteers (n = 10). VITT diagnosis was based on 4 criteria: recent AstraZeneca vaccination, positive for anti-PF4 IgG antibodies, positive in the PF4-enhanced SRA, and no prior exposure to heparin. HIT diagnosis was confirmed using the 4Ts score where all HIT patients had a clinical score of ≥4, patients had a positive commercially available PF4 enhanced heparin-dependent IgG/A/M-specific enzyme immunoassay [EIA, Immucor, WI, USA, optical density (OD) ≥ 0.45], and a positive serotonin-release assay (SRA, ≥ 20% ^{14}C-serotonin release)[27]. This study was approved by the Hamilton Integrated Research Ethics Board (HiREB) and informed written consent was obtained from all participants.

Platelet Activation Assays

Platelet activation assays were performed in the presence of heparin using the serotonin-release assay (SRA), and including a modification in which increasing doses of exogenous PF4 were added, rather than heparin (PF4-SRA).[27,28] Some assays were performed with high concentrations of unfractionated heparin (100 IU/mL), or with Fc receptor-blocking monoclonal antibody (IV.3).

Epitope mapping of antibody-binding to PF4 from VITT and HIT patients using alanine scanning mutagenesis

The full-length DNA coding sequence of human PF4 was cloned into the pET22b expression vector using restriction sites *Nde*I and *Hind*III (GenScript, Piscataway, NJ, USA). The PF4 mutants were expressed and purified as previously described[5,29]. Briefly, PF4 mutants were designed where non-alanine amino acids in wild-type PF4 were mutated to alanine and the alanine amino acids in wild-type PF4 were mutated to valine. PF4 mutants were introduced into *Escherichia coli* ArcticExpress (DE3) cells (Agilent Technologies, Santa Clara, CA, USA). Overexpression of PF4 mutant cultures were grown at 37 °C to mid-exponential phase before induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown at 37 °C for 3 hours. *E. coli* cells for each wild-type PF4 or PF4 mutant were lysed by sonication in 20 mM sodium phosphate, pH 7.2, 400 mM sodium chloride, 1.4 mM β-mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA), and 0.5% (w/v) sodium deoxycholate (Sigma-Aldrich, St. Louis, MO, USA) with 2 mM $MgCl_2$, 10 µg/mL DNaseI (Sigma-Aldrich, St. Louis, MO, USA) and EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). The supernatant was then cleared by centrifugation at 40,000 x g for 40 minutes at 4 °C and applied onto a HiTrap Q HP column (Cytiva Life Sciences, Marlborough, MAM, USA) equilibrated with 20 mM sodium phosphate, pH 7.2, 400 mM sodium chloride, 1.4 mM β-mercaptoethanol, and 5% (v/v) glycerol. The flow-through of the Q HP column was then stored at 4 °C, overnight. The following day, the serum was diluted 2-fold to yield a sodium chloride concentration of 200 mM with 20 mM sodium phosphate, pH 7.2, 1.4 mM β-mercaptoethanol, and 5% (v/v) glycerol, syringe-filtered with a 0.2 µM filter (Acrodisc, Pall) and loaded onto a HiTrap Heparin HP column (Cytiva Life Sciences, Marlborough, MAM, USA). Contaminants were eluted with 0.5 M sodium chloride and PF4 was eluted with a linear gradient from 0.5 to 2 M sodium chloride. Fractions containing pure wild-type or PF4 mutants were pooled, concentrated and buffer-exchanged to phosphate buffered saline (PBS) and 1.5 M sodium chloride. The concentration of PF4 was determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA). Protein expression and purity was assessed for each PF4 mutant using 4-18% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Measuring the effect of the 70 amino acids on the binding of anti-PF4/heparin antibodies in patient samples was analyzed similar to what has been previously described[5]. The binding of anti-PF4/heparin antibodies to wild-type PF4 and PF4 mutants was measured using a modified PF4/heparin IgG-specific EIA[5,13]. 384-well NUNC Maxisorp plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 10 µg/mL streptavidin and 1I U/mL biotinylated-heparin and blocked with phosphate buffered saline (PBS) supplemented with 3% bovine serum albumin (BSA) for 2 hours at ambient temperature. Wild-type PF4 or PF4 mutants at 5 µg/mL was then added and incubated for 1 hour at ambient temperature. Diluted patient samples (1/50 prepared in 1% BSA in PBS) in technical duplicates were added to the plates and incubated for 1 hour at room temperature. After washing, alkaline-phosphatase conjugated goat anti-human IgG (γ-chain-specific, Jackson ImmunoResearch Laboratories, Inc, Westgrove, PA, USA) was added at a 1:3,000 dilution and incubated for 1 hour at ambient temperature. Addition of 1 mg/mL p-nitrophenylphosphate (PNPP, Sigma-Aldrich, St. Louis, MO, USA) substrate dissolved in 1 M diethanolamine buffer (pH 9.6) was added for detection. The optical density (OD) was measured at 405 nm and 490 nm (as a reference) using a BioTek 800TS microplate reader (BioTek, Winooski, VT, USA) to assess binding of antibodies to wild-type PF4 and PF4 mutants. Results were reported as a percentage of loss of binding relative to wild-type PF4 binding.

Heparin inhibition of VITT anti-PF4 antibodies

Microtitre well plates (96 wells, Nunc Maxisorp, Rochester, NY, USA) were coated overnight at 4 °C with 100 µL/well of PF4 (60 µg/mL) diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6). The plates were then blocked with 200 µL/well of 3% bovine serum albumin (BSA) prepared in PBS at room temperature for 2 hours. VITT samples (n = 4) that were available were diluted 1/50 with 1% BSA in PBS and pre-incubated with increasing concentrations of unfractionated heparin (UFH, final concentration of 0.1, 0.5, 1, 5, and 100 IU/mL; Pfizer Inc., New York, NY, USA) for 1 hour at room temperature. The blocking solution was removed from the microtitre well plates and the VITT samples and heparin mixtures (100 µL/well) in technical duplicates were added to the plates and incubated for 1 hour at room temperature. The plates were washed twice with PBS/0.05% Tween 20 and thrice with PBS. Bound human IgG antibodies were detected with 100 µL/well of alkaline phosphatase conjugated goat anti-human IgG (γ-chain-specific, 1/2000, Jackson ImmunoResearch Laboratories, Inc, Westgrove, PA, USA) antibody prepared in 1% BSA in PBS. Plates were washed as before and followed with the addition of 100 µL substrate (4-nitrophenylphosphate disodium salt hexahydrate in diethanolamine (MilliporeSigma, St. Louis, MO, USA). The optical density was read at 405 nm and 490 nm (as a reference) measured using a BioTek 800TS microplate reader (BioTek, Winooski, VT, USA).

Total IgG Antibody Purification

Total IgG from two VITT patient samples were purified for further analysis of binding kinetics. A volume of 2 mL protein G-coated Sepharose beads (Thermo Fisher Scientific, Waltham, MA, USA) were washed three times with PBS at room temperature, 300 x g for 5 min. Patient samples were heat-inactivated at 56 °C for 30 min. VITT patient samples were three times diluted in PBS. The samples were then transferred to protein G Sepharose beads and incubated at room temperature for 1 hour before extensive rinsing with 30 mL PBS. Total IgG was eluted from the protein G Sepharose beads with 0.1 M glycine, pH 2.7 and neutralized by Tris buffer, pH 8.0.

Binding kinetics of VITT and HIT antibodies using biolayer interferometry (BLI)

Wild-type PF4 was labelled with biotin as previously described[30]. Briefly, wild-type PF4 and PF4 mutants were incubated with 5X the volume of Heparin Sepharose 6 Fast Flow affinity chromatography media (Cytiva Life Sciences, Marlborough, MAM, USA) for 1 hour with shaking at ambient temperature. EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA, USA) was added to the PF4 and heparin Sepharose mixture in 20 molar excess and allowed to react for 1 hour with shaking at ambient temperature. The biotinylated wild-type PF4 or PF4 mutants were eluted

from the heparin Sepharose using PBS and 2 M sodium chloride. Absorbance at 280 nm was measured using a spectrophotometer (Eppendorf AG, Hamburg, Germany) and used to calculate the concentration. Biotinylation of PF4 was checked using a streptavidin-coated anti-PF4/heparin EIA.

BLI experiments were performed using the Octet-QK Red 96 (FortéBio, Menlo Park, CA, USA). Samples or buffer were dispensed into 96-well black flat-bottom microtiter plates (Greiner Bio-one, Kremsmünster, Austria) diluted in PBS supplemented with 1% (v/v) BSA at a volume of 200 µL per well with an operating temperature maintained at 23 °C. Streptavidin-coated biosensor tips (FortéBio, Menlo Park, CA, USA) were hydrated with 1% BSA in PBS (Sigma-Aldrich, St. Louis, MO, USA) to establish a baseline prior to antigen immobilization for 60 seconds. Biotinylated recombinant PF4 (final concentration 7.5 µg/mL) alone or complexed with 0.125 IU/mL unfractionated heparin (LEO Pharma, Ballerup, Denmark) were then immobilized on the biosensor tips for 1,200 seconds at 1,000 rpm followed by a baseline re-establishment for 1,800 seconds at 1,000 rpm. Antigen-coated sensors were then reacted with heat-inactivated patient samples or purified total IgG at a 1/32 dilution in duplicate for 780 seconds at 1,000 rpm followed by a dissociation step for 3,000 seconds at 1,000 rpm. Data were analyzed using Octet® User Software version 3.1 using the 2:1 heterogenous ligand binding model. Reference values from control wells with buffer alone were subtracted and all results were aligned to the measured baseline. The binding profile response of each sample was expressed as the average wavelength/spectral shift in nanometers.

Data acquisition, statistical analysis, and reproducibility

Differences between data were tested for statistical significance using the paired or unpaired t-test and the Mann-Whitney test. *P*-values are reported as 2-tailed and a p-value of < 0.05 was considered to be statistically significant. All statistical analyses were conducted using GraphPad Prism (version 9.1.0, GraphPad Software, San Diego, USA). Experiments were repeated with technical duplicates independently two times with similar results.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

The datasets generated during and/or analysed during the current study are not publicly available to allow for commercialization of research findings but are available from the corresponding author on reasonable request.

27. Sheridan, D., Carter, C. & Kelton, J. G. A diagnostic test for heparin-induced thrombocytopenia. *Blood* **67**, 27-30 (1986).
28. Nazi, I. *et al*. Distinguishing between anti-platelet factor 4/heparin antibodies that can and cannot cause heparin-induced thrombocytopenia. *J Thromb Haemost* **13**, 1900–1907, https://doi.org/10.1111/jth.13066 (2015).
29. Huynh, A. *et al*. Development of a high-yield expression and purification system for platelet factor 4. *Platelets* **29**, 249-256, https://doi.org/10.1080/09537104.2017.1378808 (2018).
30. Huynh, A. *et al*. The role of fluid-phase immune complexes in the pathogenesis of heparin-induced thrombocytopenia. *Thromb Res* **194**, 135-141, https://doi.org/10.1016/j.thromres.2020.06.012 (2020).
31. Rubino, J. G. *et al*. A comparative study of platelet factor 4-enhanced platelet activation assays for the diagnosis of heparin-induced thrombocytopenia. *J Thromb Haemost* **19**, 1096-1102, https://doi.org/10.1111/jth.15233 (2021).

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Author contributions A. Huynh carried out the described studies, performed epitope mapping and biolayer interferometry, analyzed data and wrote the manuscript. J.G. Kelton and D.M. Arnold designed the research and wrote the manuscript. M. Daka carried out the purification of PF4 mutants, performed epitope mapping and wrote the manuscript. I. Nazy designed the research, analyzed and interpreted data and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing interests The authors declare no competing interests. A US provisional patent was submitted by Ishac Nazy, Donald M. Arnold, John G. Kelton, and Angela Huynh covering the products and methods for VITT diagnosis and differentiation from HIT using the research in this study.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41586-021-03744-4.

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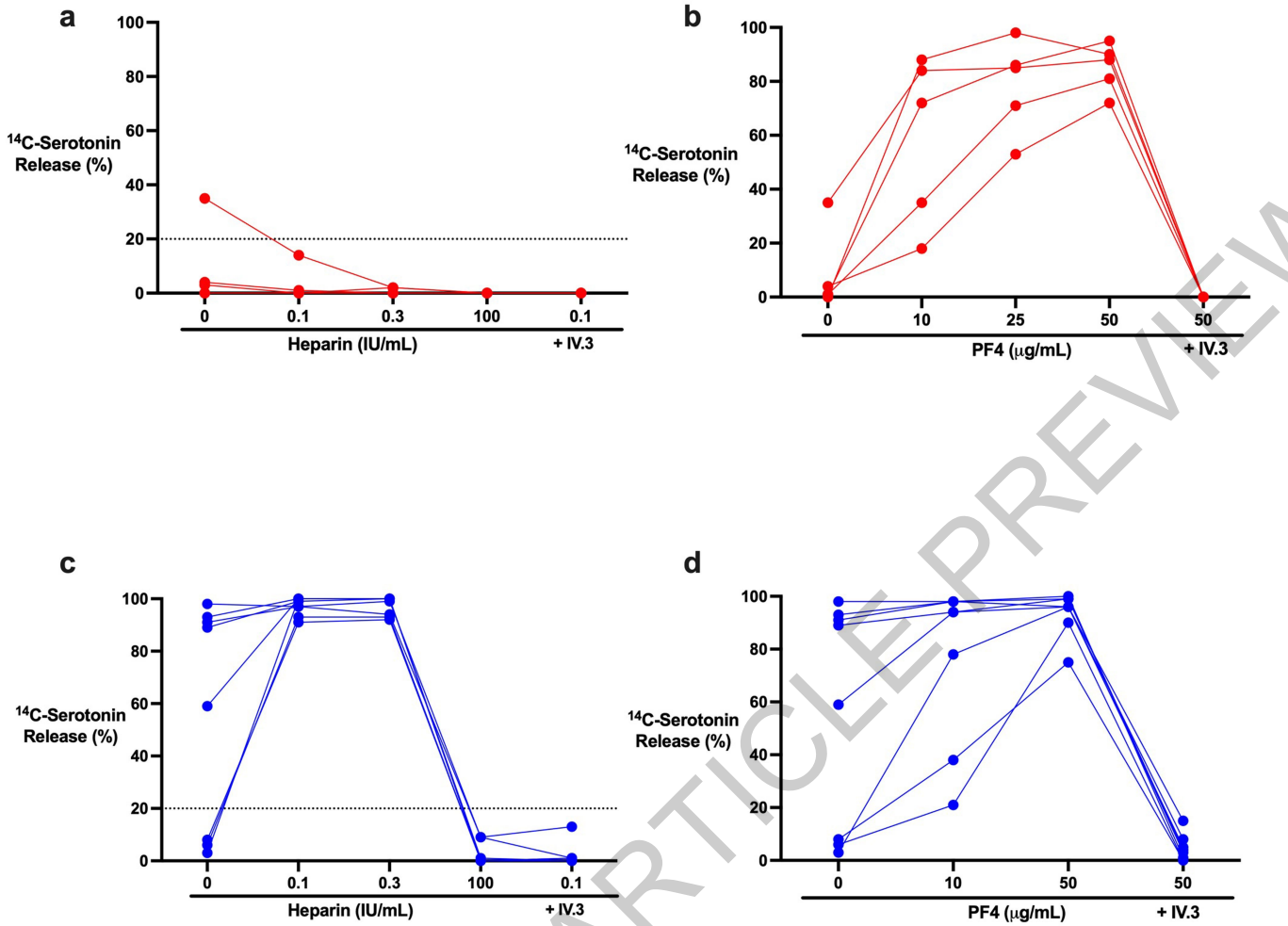
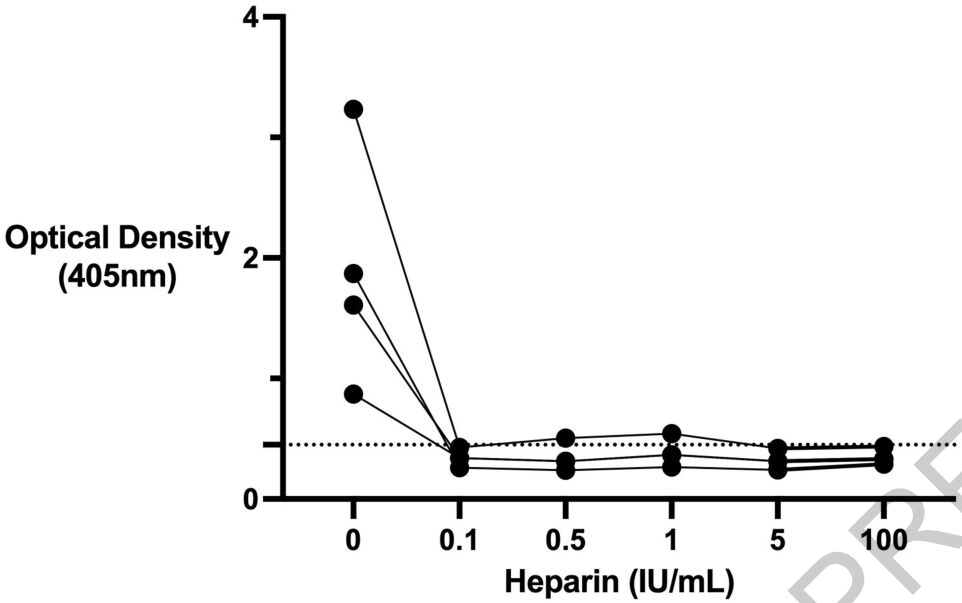
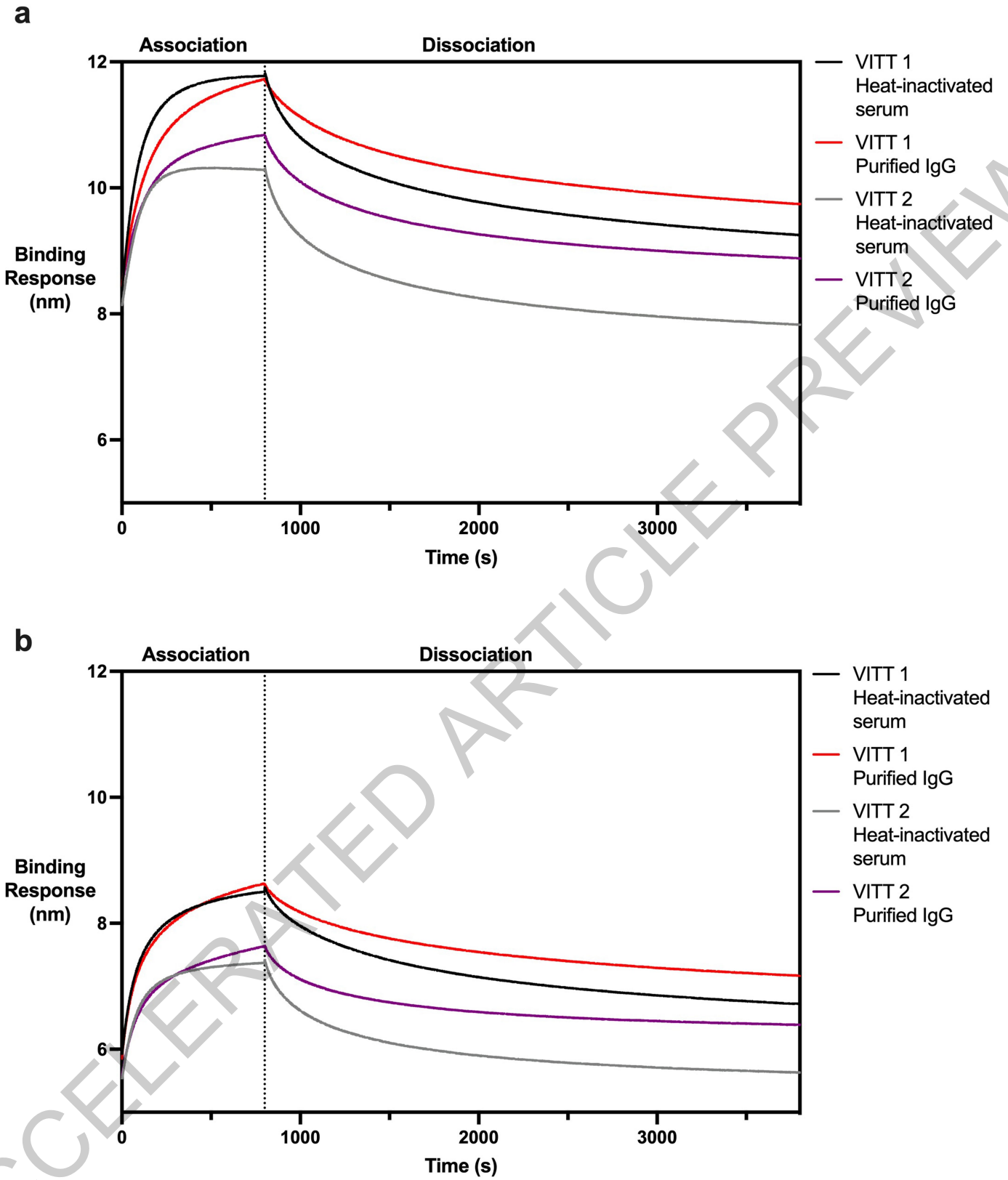
Peer review information *Nature* thanks Zaverio Ruggeri and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.



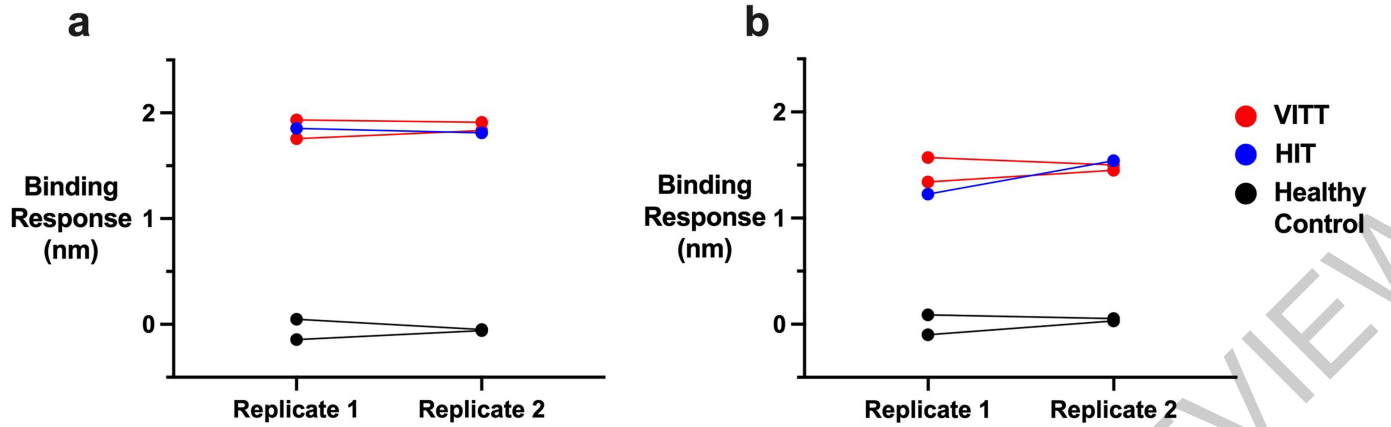
Extended Data Fig. 1 | Platelet activation (% serotonin release) of samples from A) VITT patients (n = 5, red lines) and C) HIT patients (n = 10, blue line) in the standard SRA with added heparin[27] and the same B) VITT and D) HIT samples in the PF4-enhanced SRA[28,31] with added PF4. Results demonstrated that in the standard SRA, the HIT samples activated platelets with (n = 4) or without (n = 6) heparin or with added PF4; while the VITT samples only activated platelets with the addition of PF4. Experiment was repeated with technical duplicates independently two times with similar results. All platelet activation was inhibited by the addition of IV.3 monoclonal antibody that binds the FcγRIIa receptors on platelets.



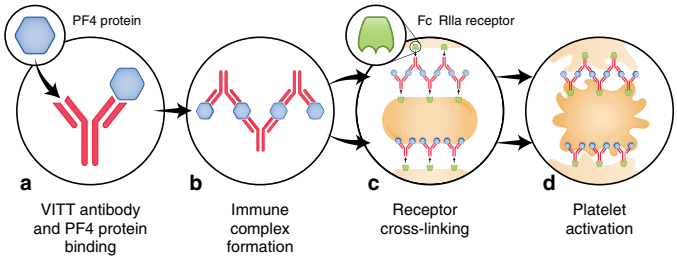
Extended Data Fig. 2 | Inhibition of VITT antibody binding to PF4 with increasing heparin concentrations. VITT samples (n = 4) were tested with increasing concentrations of heparin to investigate its effect on VITT antibodies to PF4. Experiment was repeated with technical duplicates independently two times with similar results. VITT antibody binding was disrupted by therapeutic concentrations and higher of heparin in all four VITT samples available for testing. Results are displayed as a mean of the technical replicates. An optical density (OD) > 0.45 is considered positive for anti-PF4 antibodies.



Extended Data Fig. 3 | Biolayer interferometry (BLI) curves comparing binding response in serum and purified total IgG of two VITT samples. Binding kinetics of VITT serum (n = 2) and its corresponding purified total IgG (n = 2) were measured with the Octet RED96 instrument. Experiment was repeated with technical duplicates independently two times with similar results. Association and dissociation steps are shown for **A)** biotinylated PF4 and **B)** PF4/heparin immobilized on streptavidin biosensor tips. The black lines represent the serum of VITT 1; red lines represent the purified total IgG of VITT 1. The gray lines represent the serum of VITT 2; purple lines represent the purified total IgG of VITT 2.



Extended Data Fig. 4 | Binding responses of replicate VITT, HIT, and healthy control samples. VITT (n = 2), HIT (n = 1), and healthy control (n = 2) samples were tested on different days to determine reproducibility of biolayer interferometry. Experiment was repeated with technical duplicates independently three times with similar results. Binding response against immobilized **A)** PF4 and **B)** PF4/heparin were measured as average wavelength shift in nm. Binding response for each antigen and sample was reproducible on separate assays. Red points represent VITT samples. Blue points represent the HIT sample. Black points represent healthy control samples.



Extended Data Fig. 5 | Proposed mechanism of VITT antibodies binding to and clustering PF4 tetramers, independent of heparin, and forming platelet-activating immune complexes. We postulate that **A)** VITT antibodies bind its antigen, PF4 which in turn can **B)** cluster PF4 tetramers and create platelet-activating immune complexes, in the absence of heparin. The immune complexes can be found on the platelet surface and in solution, resulting in the aligning and close proximity of the Fc-portion of these antibodies which then are able **C)** cross-link FcγRIIa receptors and **D)** lead to platelet activation.

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Reporting Summary

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☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Biolayer interferometry (BLI) data was collected using the Octet® User Software version 3.1
Data analysis	All statistical analyses were conducted using GraphPad Prism (version 9.1.0, GraphPad Software)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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The datasets generated during and/or analysed during the current study are not publicly available to allow for commercialization of research findings but are available from the corresponding author (Dr. Ishac Nazy) on reasonable request.

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☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculations were performed. The sample sizes used in the study are sufficient as both vaccine-induced thrombotic thrombocytopenia and heparin-induced thrombocytopenia are rare disorders and it is difficult to acquire clinically defined patient samples. Sample size was determined based on the availability of positive VITT samples sent for testing at the McMaster Platelet Immunology Laboratory during the month of April.
Data exclusions	No datasets were excluded.
Replication	All assays were run with technical duplicates. Each assay had the same control sample run alongside all test subjects. Two heparin-induced thrombocytopenia samples and one vaccine-induced thrombotic thrombocytopenia were run in every assay on two separate occasions. Experiments were repeated with technical duplicates independently two times with similar results.
Randomization	Allocation was not randomized. Groups were defined by clinical diagnosis. Covariates were controlled by inspection of clinical data on each patient to ensure that there was no disorder overlap or any other diagnoses that could confound the data.
Blinding	Investigators were not blinded to group allocation during analysis. Blinding was not relevant to the study as all samples were run through every assay without discrimination.

Reporting for specific materials, systems and methods

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Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Primary antibodies - VITT and HIT patient sample, healthy control samples (concentration 1/50 dilution) Secondary antibody - Alkaline-phosphatase conjugated goat anti-human IgG (γ-chain-specific, Jackson ImmunoResearch Laboratories, Inc, Westgrove, PA, USA), cat. no 109-056-098, lot no. 143072 (1/3,000 dilution)
Validation	VITT and HIT patient samples were selected from patients with confirmed diagnosis from the Platelet Immunology Laboratory. HIT patient samples were further confirmed clinically by an expert hematologist. Secondary antibody concentration used were based on previous optimizations by the lab found in the following reference: Horsewood, P. et al. Br J Haematol (1996). The secondary antibodies were validated by the manufacturer (see link https://www.jacksonimmuno.com/catalog/products/109-056-098). In summary, the antibody has been tested by ELISA and/or solid-phase absorbed to ensure minimal cross-reaction with bovine, horse, and mouse serum proteins.

Human research participants

Policy information about studies involving human research participants

Population characteristics	VITT patients (n = 5) had a mean age of 44 years (range: 35 – 72) and 2/5 were female. The time from first dose of the ChAdOx1 nCoV-19 vaccine to sample collection 14 to 40 days (mean 28 days). HIT patients (n=10) had a mean age of 69 years

	(range: 52 – 81 years) and 5/10 were female. Nine of 10 HIT patients (90%) experienced thrombosis. The time from heparin initiation to sera collection was 6 – 27 days (mean 14.3 days).
Recruitment	Sera from patients with vaccine-induced thrombotic thrombocytopenia and heparin-induced thrombocytopenia were referred to the McMaster Platelet Immunology Laboratory for confirmatory diagnosis. We tested consecutive samples as they came in. It is possible that there is self-selection bias as the samples sent for testing may be extreme cases of VITT in terms of clinical features.
Ethics oversight	Hamilton Integrated Research Ethics Board (HiREB)

Note that full information on the approval of the study protocol must also be provided in the manuscript.