Supplementary Materials

Monoclonal and oligoclonal anti-Platelet Factor 4 antibodies mediate VITT

A Kanack[1]* PhD, A Bayas[2]* MD G George[3]* MBBS, MY Abou-Ismail[4]* MD, B Singh[1] PhD, M Kohlhagen[1] BS, N Splinter[1] MS, M Christ[2] MD, M Naumann[2] MD, K Moser[5,6] MD, K Smock[5,6] MD, A Grazioli[7] MD, R Wen[8] PhD, D Wang[8] PhD, D Murray[1]¥ MD A Padmanabhan[1]¥ MBBS

* Equal contribution
¥ Corresponding authors

[1]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

[2]Department of Neurology and Clinical Neurophysiology, University Hospital of Augsburg, Augsburg, Germany

[3]Department of Medicine, University of Colorado, Aurora, CO, USA

[4]Department of Internal Medicine, University of Utah Health Sciences Center, Salt Lake City, UT, USA

[5]Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, UT, USA

[6]ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, USA

[7]Department of Medicine, National Institutes of Health, Bethesda, MD, USA and University of Maryland School of Medicine, Baltimore, MD, USA

[8]Blood Research Institute, Versiti, Milwaukee, WI 53213, USA.

Corresponding Authors:

Murray.David@mayo.edu (D.M)

Padmanabhan.Anand@mayo.edu (A.P)

VITT patient clinical histories

Patient 1's clinical course has partially been presented previously[1]. Briefly, this was a 55-year-old female with no significant past medical history who presented with conjunctival congestion, retro-orbital pain, and diplopia ten days after ChAdOx1nCoV-19 vaccination. Imaging revealed superior ophthalmic vein thrombosis (SOVT). Platelet count was 30×10^9/L (**Fig S1A**) and testing was consistent with ITP following a positive platelet suspension immunofluorescence test and positive monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay (data not shown). IgG antibodies against platelet factor 4 (PF4) were negative in lateral flow immunoassay testing (STIc Expert, Stago). Eight days after admission, the patient developed a transient, mild, right-sided hemiparesis, and aphasia. Magnetic resonance imaging (MRI) showed a new ischemic stroke in the left parietal lobe. The patient developed right-sided focal seizures adjudicated as related to the ischemic stroke which was treated successfully with levetiracetam and lacosamide. Approximately ten days post-admission D-dimer was elevated at 17,024 ng/mL FEU. Patient was treated with unfractionated heparin and dexamethasone upon admission which was associated with an increase in platelet count during the treatment period. Upon platelet recovery, anticoagulation was switched to phenprocoumon with enoxaparin bridging and the patient was discharged on day 26 post-admission. Approximately three months after initial presentation, she presented with symptoms consistent with focal seizures of the left hand. MRI revealed thrombosis in the anterior part of the superior sagittal sinus and draining veins on the left side, and in the left sigmoid sinus. D-dimer was normal, and international normalized ratio (INR) was in the therapeutic range (2 to 3). Cerebral venous sinus thrombosis (CVST) was not evident on the initial MRI at presentation. While precise time of development of CVST is unknown, one possibility includes the 2-week period after admission when platelets had not fully recovered during which anticoagulation with unfractionated heparin was measured as sub-therapeutic incidentally followed by dose adjustments.

Patient 2's clinical history is partially presented elsewhere[2]. Briefly, he was a 48-year-old male with a history of asthma who presented with a one-week history of bilateral lower extremity pain that started eleven days after receiving the Ad26.COV2.S vaccine. At presentation, he was noted to have a platelet count of 74 × 10⁹/L, fibrinogen activity of 254 mg/dL, D-dimer of 15,109 ng/mL FEU (**Fig S1B**). Venous duplex ultrasound of the lower extremities revealed bilateral occlusive and extensive deep venous thrombosis (DVTs). A computerized tomography (CT) scan of the chest revealed multiple acute bilateral pulmonary emboli in the segmental and more proximal arteries. Magnetic resonance venography (MRV) and angiography (MRA) of the brain were unremarkable. A hypercoagulable work-up did not reveal any abnormalities. A presumptive diagnosis of VITT was made, and the patient was treated with 1 g/kg of intravenous immunoglobulins (IVIG) for 2 days, 1 mg/kg of prednisone daily, and intravenous argatroban infusion drip. His anti-PF4 enzyme-linked immunosorbent assay (ELISA) (LIFECODES PF4 IgG, Immucor) performed on a pre-treatment sample demonstrated a strongly positive result of 3.323 optical density (OD) units (reference interval < 0.399), consistent with a diagnosis of VITT. Serotonin release assay (SRA; 60% release with 0.1 U/mL unfractionated heparin, 3% release with 100 U/mL unfractionated heparin) and P-selectin expression assay (PEA; 76% expression with 30 mcg/mL PF4, 0% expression with 100 U/mL unfractionated heparin) were both positive. The patient's leg and chest pain resolved, his platelet count normalized on the third day of hospitalization, and he was discharged on apixaban 10 mg twice daily. Over the subsequent month, the patient developed recurrent thrombocytopenia, with a platelet count that decreased from a peak of 205 × 10⁹/L to 107 × 10⁹/L. He maintained persistently strong positive anti-PF4 results (range: 2.4 – 2.8 OD units); however, repeat D-dimer levels, SRA, and PEA were all negative, which were interpreted to suggest that there was no further ongoing platelet activation. His thrombocytopenia appeared to moderately correlate with prednisone dose adjustments but did not respond to repeat IVIG administration.

Patient 3 was a 34-year-old woman who developed increasingly severe headaches with blurry vision, neck pain, stiffness nausea and vomiting approximately 2 weeks after receiving the Ad26.COV2.S vaccine. MRI demonstrated a large hematoma in the right posterior temporal lobe with surrounding edema and associated mass-effect with a right-to-left midline shift. Thrombi were also noted within the torcula, right transverse sinus, right sigmoid sinus, and upper internal jugular vein. At the time, she had a platelet count of 102×10^9/L (**Fig S1C**) with an elevated D-dimer of more than 40,000 ng/mL FEU and a normal fibrinogen activity (386mg/dL). She was started on IVIG (1 g/kg x 2 doses) and underwent chemical thrombolysis utilizing direct administration of TPA followed by a balloon thrombectomy. Bivalirudin was used as the anticoagulant during and after the procedure. She was also started on prednisone. She made a complete neurological recovery and was discharged ten days later with a steroid taper and on apixaban.

Patient 4 was a 46-year-old-male who received the Ad26.COV2.S vaccine. Two weeks later he experienced discomfort in his right lower extremity and a headache. A lower extremity ultrasound was negative for DVT, and CT of the head was unremarkable. His platelet count was 25×10^9/L (**Fig S1D**). Two days later he had worsening left lower extremity LLE pain and swelling, as well as ongoing headaches. Imaging was repeated; he did not have a cerebral sinus thrombosis but had developed a DVT (left popliteal vein) and pulmonary embolism. D-dimer was elevated at 28,000 ng/mL FEU and fibrinogen was 200mg/dL. He was admitted and treated with prednisone, IVIG (1 g/kg x 2 doses) and bivalirudin. He was eventually discharged on apixaban and with a prednisone taper.

Patient 5 was a 40-year-old woman who developed symptoms of headache and neck pain two weeks after receiving the Ad26.COV2.S vaccine, presented previously[3]. She was found to have an occlusive cerebral sinus venous thrombosis (CSVT) involving the left transverse sigmoid sinus and internal jugular vein. There was no brain infarction. A CT angiogram of the chest demonstrated subsegmental filling defects in the right lung, involving the posterior basal

segment and the superior segment of the right lower lobe. There were no other sites of thrombosis. On admission, she was thrombocytopenic with a platelet count of 20 000× 10^9/L with an elevated D-dimer level of 27,150 ng/ml FEU and a fibrinogen of 149 mg/dL (**Fig S1E**). She was started on bivalirudin, daily prednisone (1 mg/kg) and intravenous immune globulin (IVIG) dosed at 1 g/kg/day for two days. The patient remained clinically stable with no additional signs or symptoms. By hospital day 6, her platelets had risen to 115 000× 10^9/L, she was transitioned to and discharged on rivaroxaban and a prednisone taper. On her most recent follow up, three months later, she remains clinically stable with a platelet count of 283× 10^9/L and a D dimer of <250 ng/ml FEU.

Methods

Patient Samples

Blood samples were obtained from patients suspected of thrombotic thrombocytopenia after vaccination with ChAdOx1nCoV-19 (one patient), Ad26.COV2.S (four patients) and from patients with "classical" HIT (HIT that developed after heparin exposure), spontaneous HIT, and patients who had positive PF4/polyanion ELISA but negative SRA results after heparin exposure during cardiac surgery ("False positive", FP-HIT). Samples were drawn during acute presentation from Patients 2-5 and ~10 weeks post-acute presentation from Patient 1. Samples obtained from Patients 2-5 during the acute presentation were drawn prior to IVIg treatment, while Patient 1 was not administered IVIg. A follow-up sample from Patient 4, ~6 weeks after acute presentation, was also studied. Research studies were approved by the Institutional Review Board of Mayo Clinic.

Anti-PF4 antibody isolation

Heparin Sepharose beads (200 µL, Cytiva Lifesciences) were thoroughly washed with phosphate-buffered saline, pH 7.4 (PBS), and incubated with 200 µg of recombinant PF4 (Protein Foundry) or an equal volume of PBS (control condition). After incubation for 1hr, beads were incubated further with 500 µL of VITT/HIT/FP-HIT or 150 µL of spontaneous HIT patient sample (serum/plasma) for 1 hr. Beads were thoroughly washed with PBS, and elution from the beads was performed with 2M NaCl. The eluates (which included both PF4 and immunoglobulins) were dialyzed against PBS before being evaluated by ELISA, PEA, and mass spectrometric studies.

ELISA and Functional platelet studies

ELISA plates (Thermo Scientific) were incubated with recombinant PF4 (Protein Foundry) and Polyvinyl sulfonate (PVS, Polysciences; 9 µg/mL). Plates were washed with PBS 0.1% Tween and blocked with Superblock T20 (Thermo Scientific). Eluate samples were tested at a 1:10 dilution, while serum/plasma samples were tested at the standard 1:50 dilution. Goat anti-human IgG fc antibody (Jackson Immunoresearch) and pNPP (Sigma Aldrich) were used for colorimetric detection. Optical density was recorded at 30 minutes at 405-492 nm. The PF4-dependent p-selectin expression assay (PEA) was performed as previously described[4]. In some studies, platelets were incubated with the anti-FcγRIIa receptor monoclonal antibody IV.3 or an isotype control monoclonal antibody at a concentration of 2 ug/mL prior to PEA testing.

Liquid Chromatography Electrospray Ionization Quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF MS)

The basic method was as described elsewhere[5,6]. Immunoglobulins (Igs) from patient sera or bead eluates were isolated using camelid-derived nanobodies directed against the constant domains of gamma heavy chain, kappa light chain and lambda light chain (Thermo Fisher Scientific). 10 µL of camelid nanobody beads were incubated with 20 µL of serum or 50 µL of PF4-Heparin Sepharose (or control) bead eluate diluted into 200 µL of buffered saline (PBS) for 45 minutes at ambient temperature. Subsequently, the supernatant was removed, and the beads were washed three times with 500 µL of water. Bound immunoglobulin light/heavy chains were eluted with 100 µL of 5% acetic acid and combined with 50 µL of 100 mM dithiothreitol (DTT) in 1M ammonium bicarbonate to disassociate Immunoglobulins into separated light chain and heavy chain components. An Eksigent Ekspert 200 microLC (Framingham, MA) liquid chromatography system was used to separate immunoglobulin chains prior to ionization and detection (which removes eluted PF4 prior to mass spectrometry). The mobile phases included an aqueous phase A (100% water + 1% formic acid) and an organic phase B (90% acetonitrile + 10% isopropanol + 0.1% formic acid). 5 µL of each camelid nanobody bead elution was injected

per analysis onto a Poroshell 300SB-C3 column (1.0 mm X 75 mm) with a 5 µm particle size placed in a 60 ºC column heater. The gradient used has been described previously. The flow rate was 25 µL/minute. A SCIEX TripleTOF 5600 quadrupole time-of-flight (Q-TOF) mass spectrometer using electrospray ionization in positive ion mode was used for analysis. Data analysis was performed using Analyst TF v1.8.1 and PeakView ver. 2.2. Overexpressed Ig were inferred from the light chain +11 (m/z, mass to charge 2020 to m/z 2200) as described elsewhere[5,6]. The mass spectra of the multiply charged LC ions were deconvoluted to accurate molecular mass using the Bio Tool Kit ver. 2.2 plug-in software. The retention time of the monoclonal LC in each pre-treatment patient sample was tracked using PeakView. The instrument was calibrated every five samples using the automated calibrant delivery system (CDS). Mass measurement accuracy was estimated to be 15 ppm over the course of the analysis.

Immunofixation Electrophoresis and Immunoglobulin quantification/subtyping

Serum IFE was performed using Hydrasys 9IF gels (Sebia, Paris, France) following manufacturer's recommendations. IgG quantification and subtyping were performed using commercially available kits from Invitrogen and Thermo Fisher, respectively, following manufacturer instructions.

Supplementary Figure S1
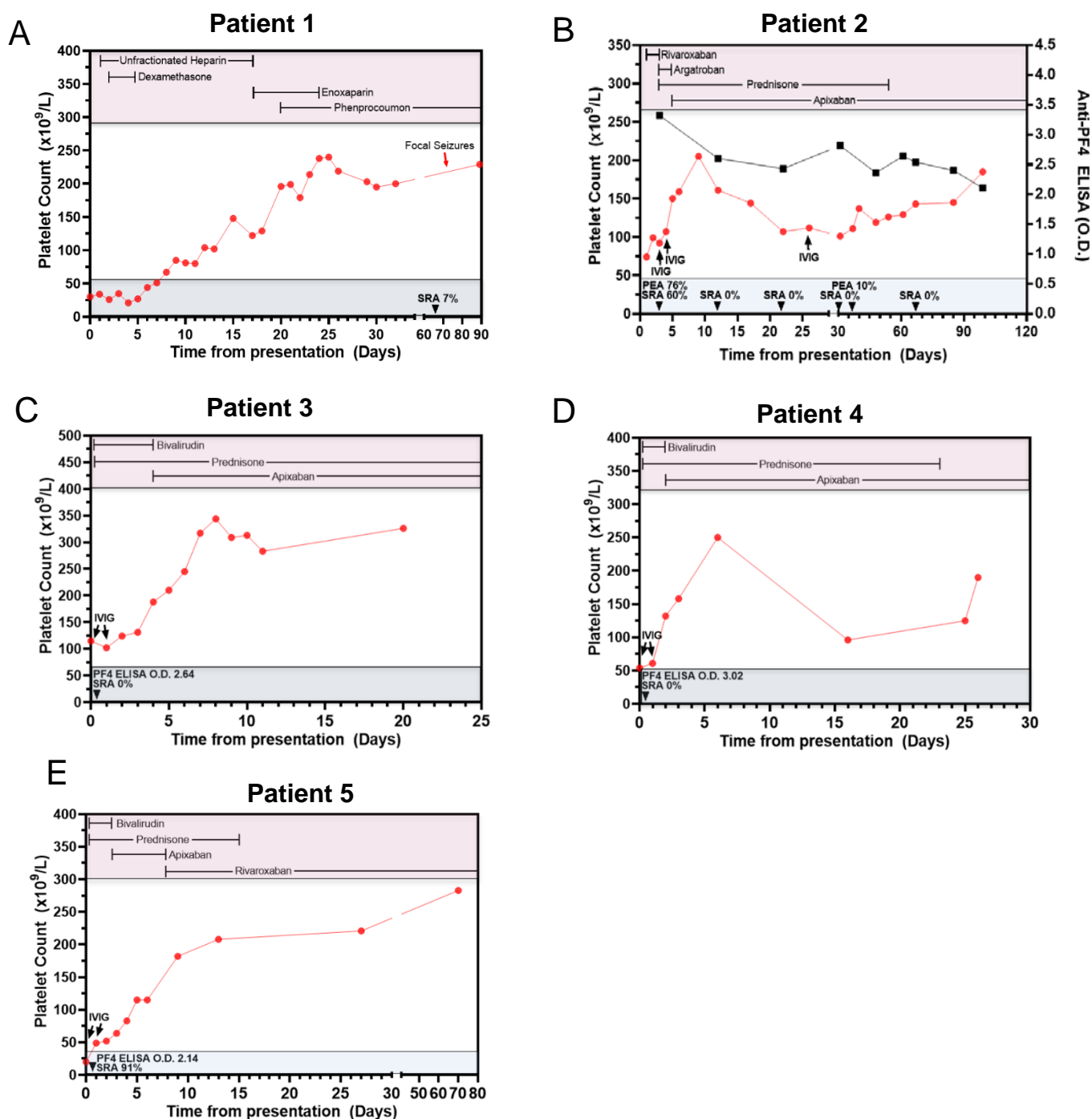
Supplementary Figure S1. Clinical Course of VITT patients. Left ordinate depicts platelet counts (red circles). Treatment interventions are displayed at the top of each panel (red box), and black arrows denote IVIG treatment (if given). For Patient 2, right ordinate denotes anti-PF4 ELISA results (black squares). Additional clinical results are presented at the bottom of each panel (gray box), with the time post-presentation denoted by black arrowheads. (**A-E**) Clinical course of Patients 1 through 5, respectively.

Supplementary Figure S2
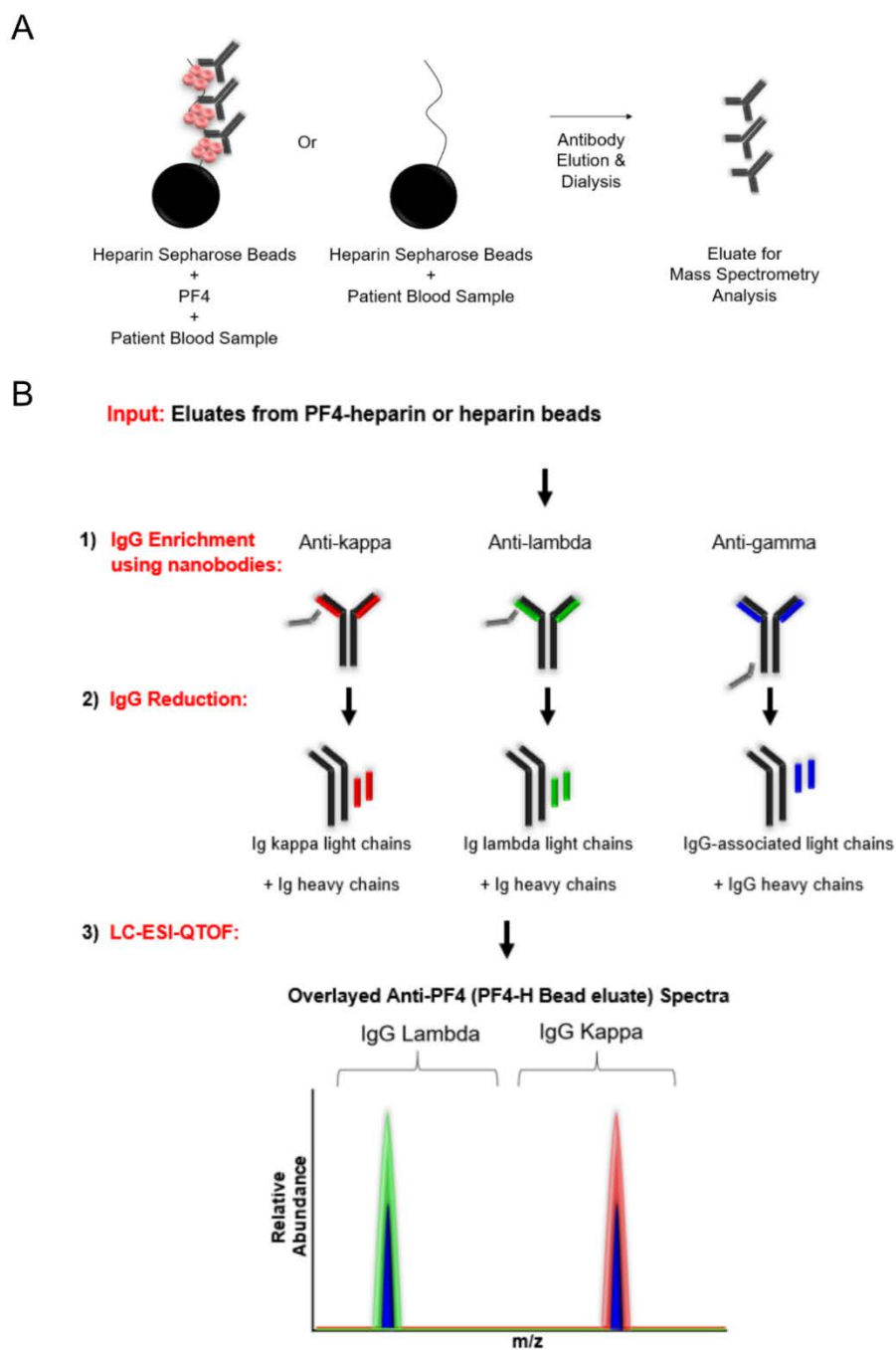
Supplementary Figure S2. Strategy for anti-PF4 antibody isolation and clonality assessment. (A) Patient sera were incubated with PF4-heparin sepharose beads, or heparin (control) sepharose beads. Antibodies were eluted by high salt concentration and dialyzed. This eluate was subject to mass spectrometric analysis. **(B)** Immunoglobulins (Igs) eluted from PF4-heparin sepharose, or heparin sepharose beads were (1) Isolated using camelid nanobodies (gray) specific for kappa light chains (red), lambda light chains (green), or gamma heavy chains

(black). IgG-associated light chains immunoenriched with anti-gamma heavy chain antibodies are shown in blue (right). Isolated Igs were then (2) reduced and (3) analyzed using liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF) to determine the antibody repertoire present. In the spectra, green represents the light chain mass to charge (m/z) distribution of all lambda containing Igs, red represents the light chain (m/z) distribution of all kappa containing Igs, and blue represents the light chain (m/z) distribution of kappa and lambda light chains associated with an IgG heavy chain. Spectra are overlaid to confirm the type of light chain and antibody isotype. In this example, the anti-PF4 antibody is biclonal, with one IgG lambda and one IgG kappa monoclonal antibody.
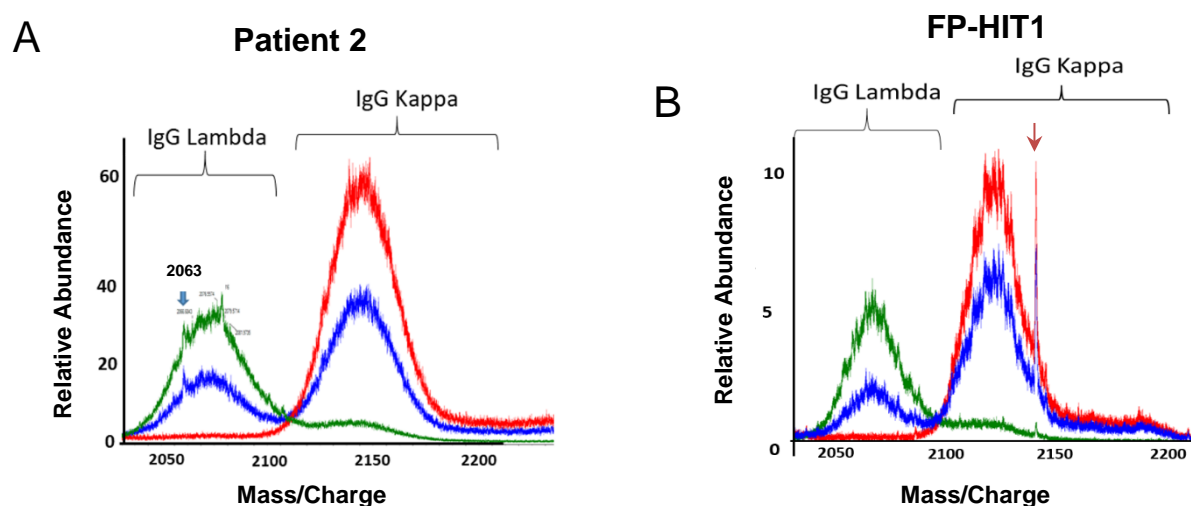
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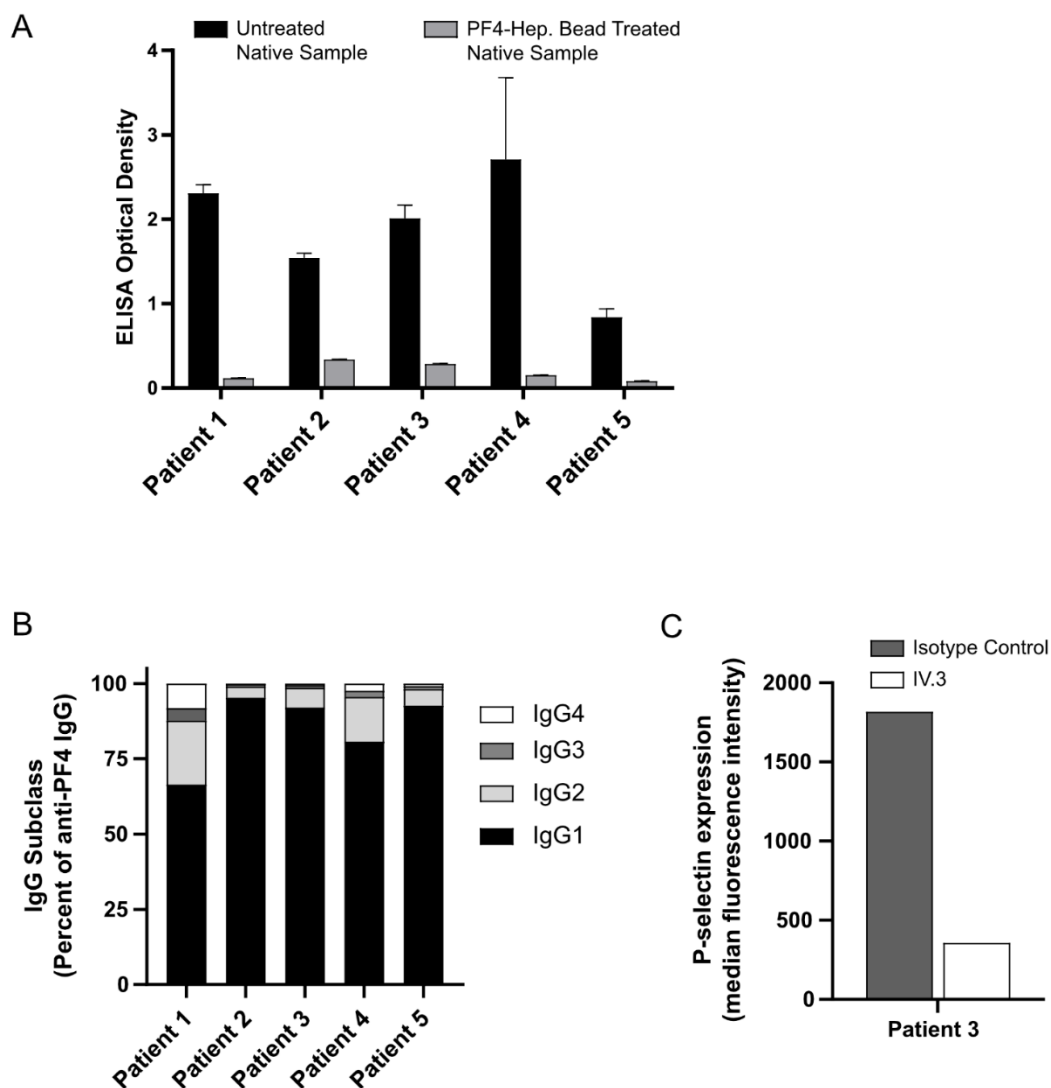
Figure S3. Affinity purification effectively depletes anti-PF4 antibodies from the native sample and VITT antibodies are primarily of the IgG1 subclass. (**A**) Native sera/plasma samples from five VITT antibodies were tested in PF4/polyanion ELISA before (black bars) and after affinity purification (grey bars) with PF4-heparin sepharose beads. Means and SD (n=3) are presented. (B) IgG subclass composition of the isolated anti-PF4 antibodies is shown. (C) VITT antibody-mediated platelet activation is blocked by treatment of platelets with monoclonal antibody IV.3, while it is unaffected by a murine isotype control antibody.
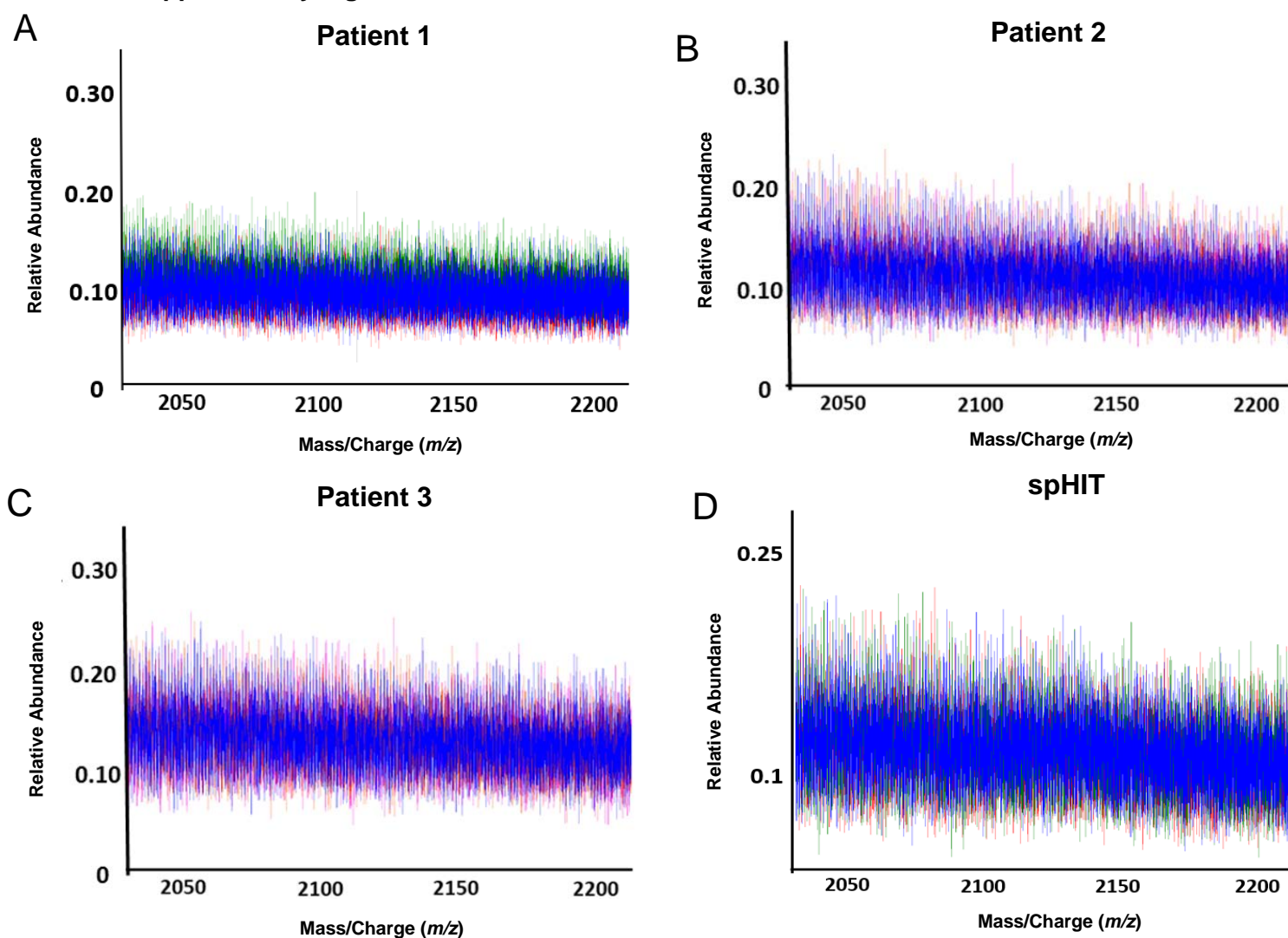
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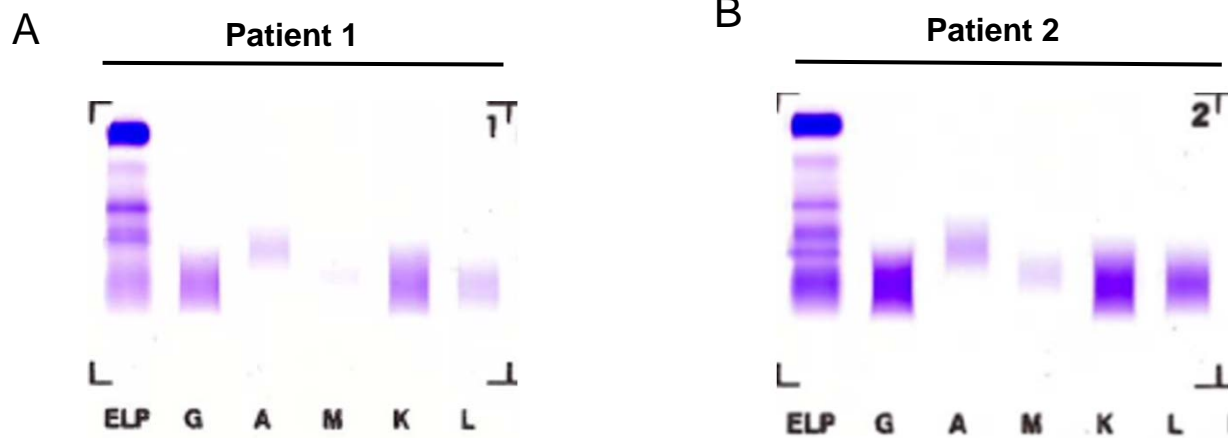
Supplementary Figure S4. Immunofixation electrophoresis of native VITT samples does not demonstrate mono/oligoclonal bands. Serum samples from Patient 1 **(A)** and Patient 2 (**B**) were run by electrophoresis with immunofixation to detect IgG, IgA, IgM, kappa, and lambda light chains following manufacturer instructions. In order, the gel lanes are presented as protein fixative (ELP), IgG (G), IgA (A), IgM (M), kappa light chain (K), and lambda light chain (L).

Supplementary Figure S5

A **Patient 2**

B **FP-HIT1**

Supplementary Figure S5. Mass Spectrometric evaluation of VITT and FP-HIT native sera. Displayed are LC-ESI-QTOF MS +11 light chain distributions from native sera of VITT Patient 2 (**A**) and FP-HIT1 (**B**). In the spectra, green represents the distribution of all lambda containing immunoglobulins (Igs), red represents the distribution of all kappa containing Igs, and blue represents the light chain distribution of kappa and lambda light chains associated with an IgG heavy chain. The X-axis depicts mass/charge ratios, and Y-axis depicts the relative abundance of the antibodies identified. The arrow in (**A**) depicts the monoclonal antibody identified in this patient after anti-PF4 isolation (**Fig 4B**), while the arrow in (**B**) shows an irrelevant monoclonal antibody in FP-HIT1 not pulled down with PF4-heparin sepharose beads (**Fig 5E**).

Supplementary Figure S6

A
Patient 1

B
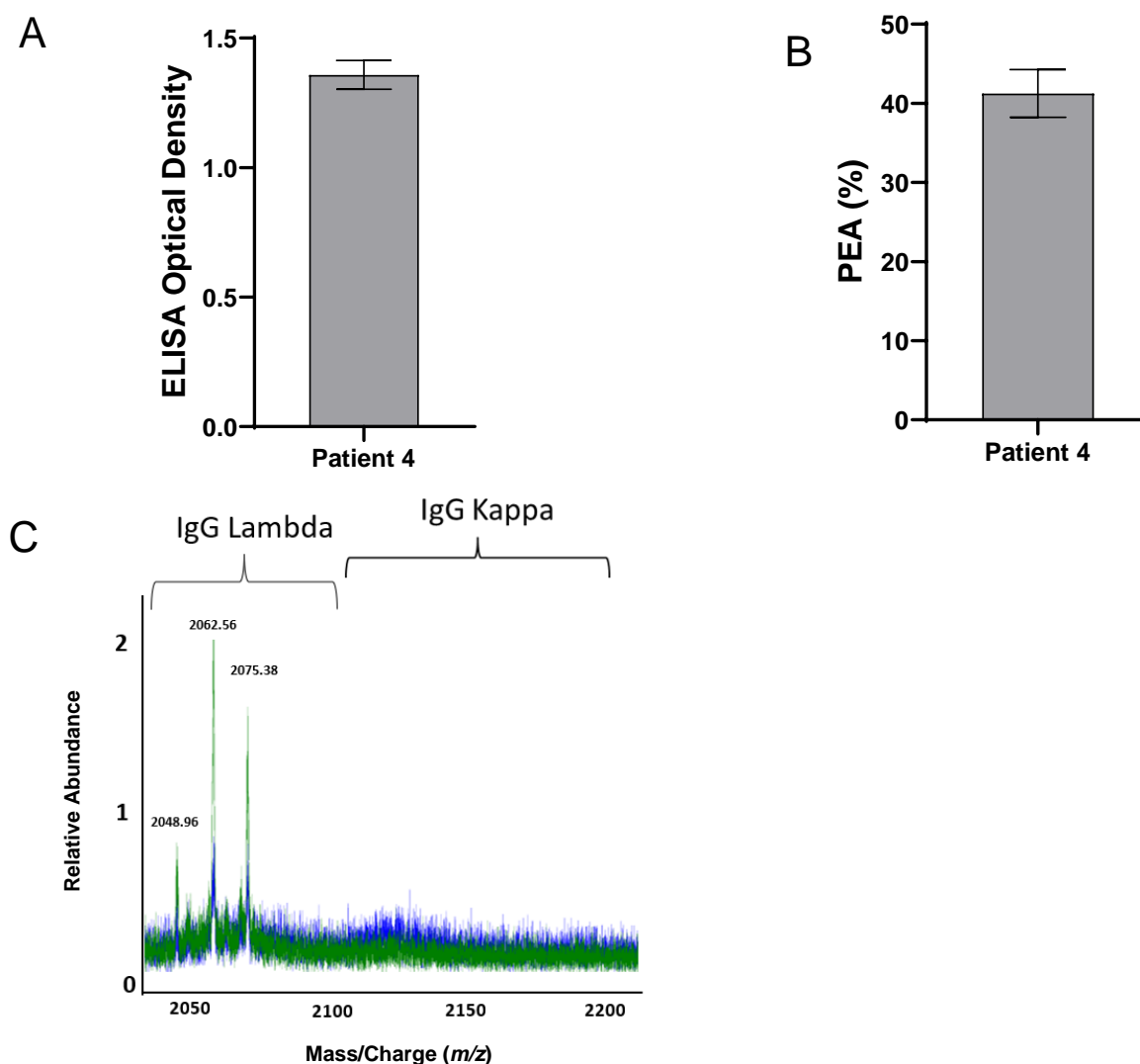## Patient 2

C
Patient 3

D
spHIT

Supplementary Figure S6. Elution from heparin-sepharose beads incubated with VITT and spontaneous HIT patient samples showed no Immunoglobulins in LC ESI QTOF MS. Displayed are LC-ESI-QTOF MS +11 light chain distributions from anti-PF4 antibodies isolated patients with VITT (**A-C**) and Spontaneous HIT (spHIT) (**D**). In the spectra, green represents the distribution of all lambda containing immunoglobulins (Igs), red represents the distribution of all kappa containing Igs, and blue represents the light chain distribution of kappa and lambda light chains associated with an IgG heavy chain. The X-axis depicts mass/charge ratios, and Y-axis depicts the relative abundance of the monoclonal/oligoclonal antibody identified. No Igs were seen.

Supplementary Figure S7

A

B

C

Supplementary Figure S7. VITT antibodies are persistent. Patient 4 sample was obtained and tested ~6 weeks after initial acute presentation. **(A)** Antibody binding to PF4/Polyvinylsulfonate (PVS; light gray) was evaluated by ELISA. **(B)** Activation of VITT patient antibodies in the PF4-dependent P-selectin Expression Assay (PEA) was examined. Mean and 1 SD are shown (n=3). **(C)** Displayed are LC-ESI-QTOF MS +11 light chain distributions from anti-PF4 antibodies isolated from this patient. In the spectra, green represents the distribution of all lambda containing immunoglobulins (Igs), red represents the distribution of all kappa containing Igs, and blue represents the light chain distribution of kappa and lambda light chains associated with an IgG heavy chain. The number listed above peaks indicates the identified light chain's mass/charge (m/z) ratio. The X-axis shows mass/charge ratios and Y-axis depicts the relative abundance of the oligoclonal antibodies identified.

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