Supplementary Appendix

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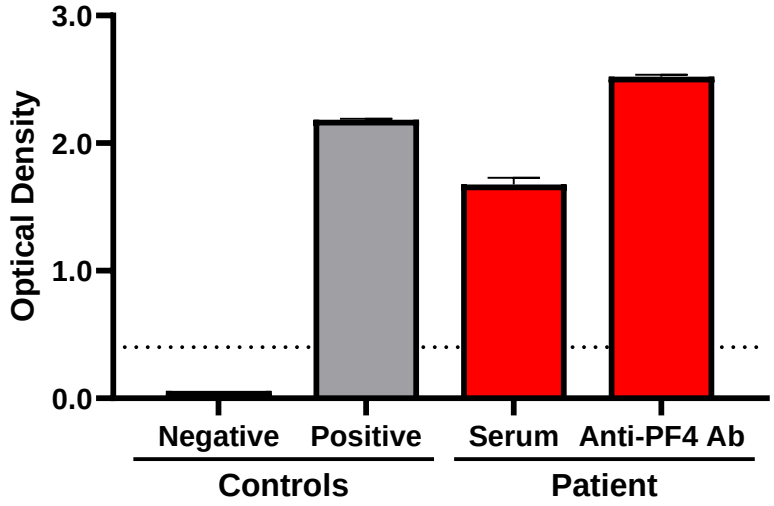
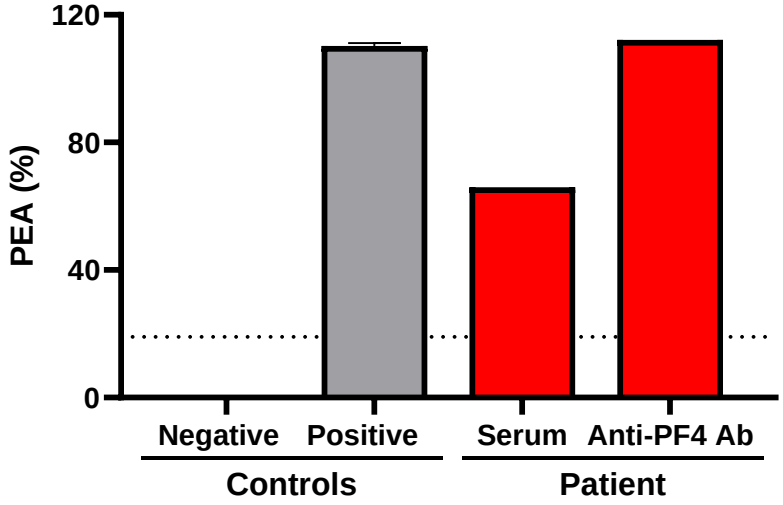
S1A



S1B



Supplementary Figure 1. Native sample and eluate from PF4/heparin sepharose beads were tested in **(A)** Lifecodes PF4 IgG ELISA (Immucor), and **(B)** PF4-dependent P-selectin expression assay (PEA). The dotted line represents the positive cut-off of the assays. Means + 1 standard deviation of duplicate determinations are presented in **(A)** and singleton determinations in **(B).**

Methods

Patient Samples and diagnostic testing

Blood samples were obtained from the patient following informed consent. Research studies were approved by the Institutional Review Board of Mayo Clinic.

Anti-PF4 antibody isolation

Anti-PF4 antibodies were isolated as described recently[1,2]. Briefly, heparin sepharose beads (200 µL, Cytiva Lifesciences) were washed with phosphate-buffered saline, pH 7.4 (PBS), and incubated with 200 µL (1 mg/mL) of recombinant PF4 (Protein Foundry) for one hour. Beads were then incubated with 250 µL of patient sample (serum) for one hour. Beads were thoroughly washed with PBS, and elution from the PF4/heparin sepharose beads was performed with 250 µL of 2M NaCl. Eluates were dialyzed against PBS before being evaluated by HIT enzyme-linked immunosorbent assay (ELISA), PF4-dependent P-selectin expression assay (PEA), and mass spectrometric studies.

HIT ELISA

Patient samples were evaluated in Lifecodes PF4 IgG (Immucor) ELISA, an FDA-approved in vitro diagnostic assay that employs PF4-polyvinyl sulfonate (PVS) targets. Lifecodes PF4 IgG (Immucor) was used according to the manufacturer's instructions. In brief, patient serum/plasma was incubated with PF4-PVS coated platelets, stringently washed, and incubated with an alkaline phosphatase labeled anti-human IgG antibody. After antibody incubation, pNPP substrate was added and colorimetric detection was performed.

Functional platelet studies

The PEA was performed as previously described[3,4]. Briefly, prostaglandin E1 was added to citrated whole blood obtained from healthy volunteers at a final concentration of 50 ng/mL and centrifuged at 200 x g for 15 minutes to obtain platelet-rich plasma (PRP). Platelets were then isolated from PRP by centrifugation at 1,000 x g for 15 minutes, after which the platelet pellet was resuspended in phosphate-buffered isotonic saline (pH 7.4) supplemented with 1% bovine

serum albumin. Platelets (1 x 10^6) were treated for 20 minutes at room temperature with PF4 (37.5 µg/mL). After PF4 incubation, ten microliters of patient sample was added to 40 µL of PF4 treated platelets and incubated for one hour at room temperature, yielding a final PF4 concentration of 30 µg/mL. Fluorescently labeled anti-P-selectin (monoclonal antibody HB-299, ATCC) and anti-GPIIIa (monoclonal antibody HB-242, ATCC) antibodies were added to the samples for 20 minutes, before being analyzed by flow cytometry. Platelet events were gated for GPIIIa positivity in flow cytometry, and P-selectin expression (median fluorescence intensity, MFI) was recorded. The SRA was performed in various CLIA-approved reference testing laboratories, as determined by the treating physician.

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

The basic method used for antibody analysis has been previously described[5,6]. Immunoglobulins (Igs) from patient sera or bead eluates were isolated using camelid-derived nanobodies against the constant domains of human Ig gamma heavy chain, kappa light chain, or lambda light chains (Thermo Fisher Scientific). In summary, 100 µL or 50 µL of camelid nanobody beads were incubated with 10 µL of serum or 100 µL of PF4-Heparin Sepharose eluate, respectively, diluted into 200 µL of buffered saline (PBS) and incubated for 30 minutes at ambient temperature. Subsequently, supernatants were removed, and the beads were washed three times with 500 µL of water. Bound Ig from light and heavy chains of serum and anti-PF4 antibody eluate were eluted using 60µL or 20µL of 5% acetic acid respectively. Following a 5-minute incubation, eluted Igs were reduced using dithiothreitol (DTT) in 1M ammonium bicarbonate (2:1; v:v) to disassociate immunoglobulins and separate light chain from heavy chain Ig components. An Agilent 1290 Infinity II liquid chromatography (LC) system was used to separate immunoglobulin chains prior to ionization and to remove eluted PF4 before analysis using a SCIEX Zeno time-of-flight (TOF) 7600 mass spectrometer (MS). Ten microliters of each camelid nanobody bead eluate were injected per analysis onto a Poroshell 300SB-C3 column

(2.1 mm X 75 mm) with a 5 µm particle size placed in a 60 ºC column heater. 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), and the flow rate was 300 µL/min. The light chains are typically eluted during a 4.5-minute gradient from 27%B to 32%B. The diverter valve was used to direct 10.35 minutes of the gradient into the MS; otherwise, the LC was diverted to waste. The MS, using positive electrospray ionization, was run using intact protein workflow; CUR 30, CAD 7 GAS1 35, GAS2 30, and temperature 500 °C. TOF MS data from collected from 600 to 2500 m/z; DP 175 and CE 10. Data analysis was performed using Analyst TF v1.8.1 and PeakView ver. 2.2. Overexpressed immunoglobulins were inferred from the light chain +11 (m/z, mass to charge 2020 to m/z 2200) as described elsewhere[5,6]. The retention time of the monoclonal light chain in each patient sample was tracked using PeakView. The mass spectra of the multiply charged light chain ions were deconvoluted to obtain an accurate molecular mass using the Bio Tool Kit ver. 2.2 plug-in software. The instrument was calibrated every five samples using an automated calibrant delivery system (CDS). Mass measurement accuracy was estimated to be 15 ppm for the duration of the analysis.

References

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