Figure S1: Activation pattern of VITT sera
Platelet activation of the 16 tested VITT sera in PIFPA with all 4 donors (D1-D4).
**Figure S2:** Colored depiction of the results from the tested VITT sera and unvaccinated controls that were used to define the cut-off

**Figure S3:** Titration of PF4 with unvaccinated controls and VITT patients
Detailed method protocols

Platelet factor 4-induced platelet activation test (PIPA)

Platelet preparation

Platelets were purified from ACD-A anticoagulated whole blood obtained from healthy donors who did not take antiplatelet medications or non-steroidal anti-inflammatory drugs (NSAIDs) during the previous 10 days, as described.\textsuperscript{7,8} In brief, platelet-rich plasma (PRP) was centrifuged (7 minutes at 650 g, without brake) and the platelet pellet washed with Tyrode’s buffer containing 0.35% BSA (albumin bovine Fraction V, Serva, Germany), 0.1% glucose (B. Braun, Germany), 2.5 U/mL apyrase (Sigma Aldrich, Germany), 1 U/mL hirudin (Canyon Pharmaceuticals, Switzerland), pH 6.3. After a further centrifugation (7 minutes at 650 g, without brake), the final platelet pellet was resuspended in a bicarbonate-based suspension buffer consisting of 0.137 M NaCl, 0.027 M KCl, 0.012 M NaHCO\textsubscript{3}, 0.42 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.35% BSA, 0.1% glucose, 0.212 M MgCl\textsubscript{2}, 0.196 M CaCl\textsubscript{2}, pH 7.2 and adjusted to 300,000 platelets/µL.

A video tutorial how to prepare washed platelets is available at: https://www.youtube.com/watch?v=hFs-_85YJX4

PIPA test assessment and interpretation

Heat-inactivated (56 °C, 30 min) patient serum (20 µL) and washed platelets (75 µL) were incubated in a microtiter plate (Greiner, Austria) with either buffer, 0.2 aFX U/mL low-molecular-weight heparin, reviparin (Abbott, Germany; if reviparin is not available enoxaparin can be used), 100 IU/mL unfractionated heparin (ratiopharm, Germany) - as described for heparin-induced platelet activation (HIPA) test or 10 µL platelet factor 4 solution (10 µg/mL, final conc., Chromatec, Germany) in the presence and absence of the FcγIIa receptor- blocking antibody, IV.3 (5 µL added to 75 µL platelets, obtained by cell supernatant, cell line ATCCHB-217, Biometec GmbH). To avoid any effect of thrombin, to all conditions (with the exception of the 100 IU/mL reaction well) hirudin (5 U/mL) was added. If the effect of intravenous immunoglobulin (IVIG) should be tested, patient serum should be co-incubated with platelets in the presence of IVIG at a concentration of 10 mg/mL before PF4 10 µg/mL is added.
The microtiter plate was incubated (45 min, RT) on a magnetic stirrer (1000 rpm) with two steel spheres (2 mm diameter, SKF, Mercateo). The transparency of the suspension was assessed using an indirect light source every 5 min. The PIPA test was considered positive in the presence of platelet aggregation shown by clearance of the solution and visible aggregates – obtained within 30 min in at least 2 of 3 (or 2 of 4) test cells - in the presence of PF4 alone (or in buffer as well).

**Platelet factor 4-induced flow cytometry-based platelet activation test (PIFPA)**

Citrated whole blood was obtained from 4 healthy donors who did not take anti-platelet medication or non-steroidal anti-inflammatory drugs (NSAIDs). From each donor 198 µL whole blood were spiked with 2 µL Hirudin 5400 U/mL (Canyon Pharmaceuticals, Switzerland; if hirudin is not available other thrombin inhibitors like PPACK can be used). In a subset of experiments FcγRIIa blocking antibody IV.3 was added in a ratio of 1:16 to whole blood and incubated for 30 min at 37°C before starting the main experiment. Human platelet factor 4 (Chromatec, Germany) was diluted in 1x PBS w/o Ca²⁺, Mg²⁺ (pH 7.4, PAN Biotech, Germany) to 20 µg/mL (=5 µg/mL final concentration in the assay) or 80 µg/mL (=20 µg/mL final concentration in the assay). 5 µL whole blood spiked with hirudin were transferred to 5 mL polypropylene tubes (Sarstedt, Germany) and incubated for 20 min at 37°C with 2.5 µL of either PF4 20 µg/mL (5 µg/mL final concentration in assay), PF4 80 µg/mL (20 µg/mL final concentration in assay) or the same amount of 1x PBS as control. Afterwards, 2.5 µL undiluted, heat inactivated (56°C, 30 min) sera (from patients with VITT, asymptomatic vaccinated donors being positive or negative in the anti-PF4/heparin EIA, unvaccinated donors, or patients with HIT) were added to each tube and incubated for further 20 min at 37 °C.

For this assay, preferentially heat inactivated serum should be used. Serum can be used fresh or stored frozen. Repetitive freezing and thawing should be avoided. Serum can be thawed at room temperature, but thawing at 37°C in a water bath should be preferred. Serum should be heat inactivated (56°C, 30 min) to avoid any impact of thrombin and complement. If serum is not available, citrated plasma might also be used (EDTA or heparin anticoagulated plasma should be avoided), but data are still very limited. Plasma should also be heat inactivated (56°C, 30 min) and then centrifuged in an 1.5 mL tube (10,000 xg, 10
min). The supernatant is then carefully transferred into a new tube and the fibrin/fibrinogen pellet discarded.

Each sample was incubated with 1 µL CD61-PE (clone SZ21, Beckman Coulter, USA) and 1 µL CD62P-PE-Cy5 (Becton Dickinson, USA) for 10 min at room temperature in the dark, followed by a fixation step of 20 min at room temperature with 2% PFA (Morphisto, Germany). For a washing step 500 µL PBS was added to each sample before centrifugation with 650 xg, 7 min, room temperature). The supernatant was discarded, the pellet was resuspended in 500 µL 1x BD FACS Lysing Solution (Becton Dickinson, USA, diluted from 10x stock solution in A. dest.) and incubated at least 10 min before starting flow cytometry using a Cytomics FC500 (Beckman Coulter, USA).

Statistical analyses were performed with GraphPad Prism version 7.04 software. Platelets were positively gated using CD61-PE and activation was determined by the degree of granule release measured using CD62P-PE-Cy5 antibody and given as mean fluorescence intensity (MFI) of the CD62P positive gated events multiplied by the percentage of gated platelets. The gate was set accordingly by a not activated negative control containing PBS instead of serum (not shown). A final concentration of 20 µM thrombin receptor activating peptide (TRAP-6, Hart Biologicals, UK) served as a positive control for each whole blood sample (not shown). Platelet activation for each serum was analyzed as the median activation result from the four whole blood samples. Statistical significance was determined by using unpaired t-tests.