## SUPPLEMENTARY MATERIAL

# Anti-PF4 immunothrombosis without proximate heparin or adenovirus vector vaccine exposure

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## SUPPLEMENTARY METHODS

### Prospective Cohort with Incident Stroke study (PROSCIS-B):

The prospective cohort with incident stroke (PROSCIS) study Berlin is a prospective hospital-based cohort study conducted at three tertiary stroke centres in Berlin, Germany (clinicaltrials.org/NCT01363856; PMID: 22928669). PROSCIS-B is conducted by the Center for Stroke Research Berlin, Charité University Hospital, Germany, and enrolled patients between January 2010 and June 2013. The study enrolled patients aged 18 years or older with first ever acute stroke within the last 7 days. Key inclusion criteria included: (1) a diagnosis of ischaemic stroke, primary intracranial haemorrhage, or venous sinus thrombosis according to the WHO criteria, and (2) written informed consent as documented by patient or legal guardian prior to study participation. Key exclusion criteria included: (1) prior stroke (definition according to WHO criteria); (2) brain tumour or brain metastasis; and (3) participation in an intervention/AMG study. Baseline assessments included: a structured interview that collected information about demographic variables, living situation, functional pre-stroke outcome, lifestyle habits, health and family history, as well as medication before stroke provided by the patient or the next of kin and cognitive function before and after stroke. Clinical examinations included anthropometric measures and stroke severity. Vascular and cardiological examinations included standardized physiological measures of blood pressure, electrocardiography, and brain and vessel imaging. All participants provided informed

consent and protocols were approved by the institutional review board. Ischemic stroke subtypes were determined according to TOAST criteria based on relevant clinical and imaging data by stroke physicians.

**Anti-PF4/heparin IgG enzyme-immunoassay (EIA)**

Binding of immunoglobulin G anti-PF4 antibodies from sera of VITT patients to PF4/heparin complexes will be measured by a solid phase PF4/heparin EIA performed in flat-bottomed microwell plates (Cat. No. 478042 Thermo Scientific, CovaLink). PF4/heparin complexes of 0.5 IU/mL unfractionated heparin (UFH; Heparin-Natrium 25000 IE/ 5 mL, Ratiopharm) and 20 µg/mL PF4 will be formed in coating buffer (50 mM NaH2PO4, 0.1% NaN3) at RT for 1 h and will incubate for seven days at 4°C. Prior to use, complexes will be washed five times with washing buffer (150 mM NaCl, 1% Tween20 pH 7.5). 100 µL/microwell of serum samples (diluted to 1:200 if not indicated otherwise), in sample diluent (0.05 M NaH2PO4, 0.15 M NaCl, 7.5% goat normal serum, pH 7.5) will be incubated for 60 min, at RT and then washed five times. Horseradish peroxidase-conjugated goat antihuman IgG (Jackson ImmunoResearch Europe Ltd, Ely, UK) will be added (1:15,000 dilution in sample diluent). Binding of human IgG will be detected by adding chromogenic tetramethylbenzidine substrate (100 µL/microwell; Kementec, Taastrup, Denmark). At 60 min post-incubation, the chromogenic substrate reaction will be stopped with 1 M H2SO4 (100 µL/microwell) for 10 min at RT, and optical absorbance will be measured at 450 nm (reference: 620 nm, Tecan, Männedorf, Switzerland) within 10 min. Blank measurements will be subtracted from each sample measurement. For quality control each lot of PF4 is tested with a panel of 20 different antibodies containing anti-PF4/heparin antibodies with known reactivity. Each lot of PF4/heparin complex coated plates is controlled with the same quality panel of known anti-PF4/heparin antibodies for sufficient reactivity. Each EIA test is controlled with a known weak positive control and a known negative control.

**PF4-dependent platelet activation assay (PIPA test)**

*Platelet preparation*

Platelets will be 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 demonstrated in the video tutorial available at: https://www.youtube.com/watch?v=hFs-_85YJX4

Platelet-rich plasma (PRP) will be 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 will be resuspended in a bicarbonate-based suspension buffer consisting of 0.137 M NaCl, 0.027 M KCl, 0.012 MNaHCO3, 0.42 mM NaH2PO4, 0.35% BSA, 0.1% glucose, 0.212 M MgCl2, 0.196 M CaCl2, pH 7.2 and adjusted to 300,000 platelets/µL.

*PIPA test assessment and interpretation*

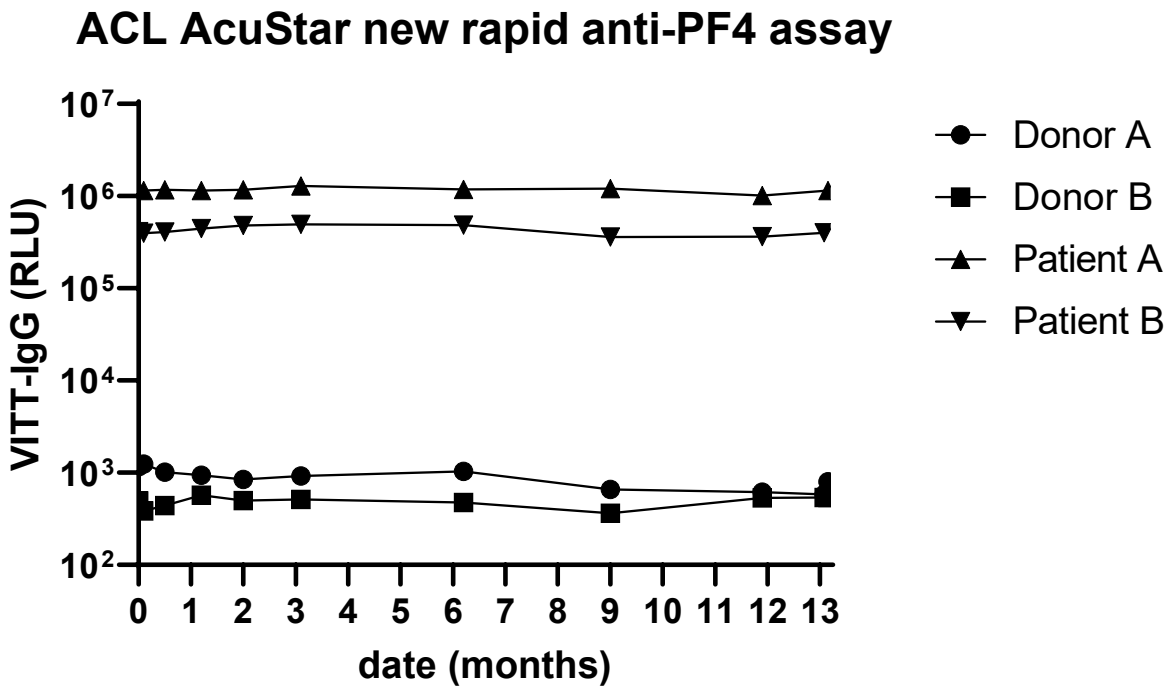
Heat-inactivated (56 °C, 30 min) patient serum (20 µL) and washed platelets (75 µL) will be incubated in a microtiter plate (Greiner, Austria) with either buffer, 0.2 aFX U/mL low-molecular-weight heparin, reviparin (Abbott, Germany; if reviparin is not available enoxaparin can be used; HIPA), 100 IU/mL unfractionated heparin (ratiopharm, Germany), or 10 µL platelet factor 4 solution (10 µg/mL, final conc., Chromatec, Germany; PIPA) 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 heparin reaction well) hirudin (5 U/mL) will be added.

The microtiter plate will be incubated (45 min, RT) on a magnetic stirrer (1000 rpm) with two steel spheres (2 mm diameter, SKF, Mercateo). The transparency of the suspension will be assessed using an indirect light source every 5 min. A positive result was defined as activation of platelets (lag time ≤30 min) of at least two of three different donors and inhibition at high heparin concentrations (100 IU/mL). Reactivity lag time of >20-30 min was defined as weak, >10-20 min as moderate, and ≤10 min as strong reactivity.

For quality control each platelet preparation is incubated with low concentrations of collagen to secure sufficient platelet reactivity. Each test is controlled by a known serum containing anti-PF4 antibodies causing platelet activation within 15 to 25 minutes.

A positive result was defined as activation of platelets (lag time ≤30 min) of at least two of three different donors and inhibition at high heparin concentrations (100 IU/mL). Reactivity lag time of >20-30 min was defined as weak, >10-20 min as moderate, and ≤10 min as strong reactivity. For sera that tested strongly positive with buffer, we performed dilutions with saline until buffer reactivity became negative and retested them with heparin 0.2 aFXa U/ml and PF4 10 µg/ml.

Supplementary Figure 1




**Legend Supplementary Figure 1:**
Two VITT and two donor samples were tested over the course of a year with the new rapid anti-PF4 assay. Positive sample deviation mean accounted for 1.1 ± 9.9%.