SUPPLEMENTARY APPENDIX

Structural analysis of ischemic stroke thrombi: histological indications for therapy resistance

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Supplemental Methods

Hematoxylin and Eosin

Staining with hematoxylin and eosin was performed via standard procedures (H&E; HT110216, Sigma-Aldrich, St. Louis, MO). In brief, sections were stained for one minute at RT with hematoxylin and eosin, respectively. Hematoxylin stains basophilic components such as nuclei in purple, whereas eosin stains acidophilic components such as fibrin/platelet aggregates in pink and red blood cells in red.

Martius Scarlet Blue

Martius Scarlet Blue (MSB) staining was used to visualize red blood cells (yellow), fibrin (dark pink to red) and collagen (blue). Sections were immersed in Bouin's fluid (HT-10132; Sigma-Aldrich) at 56°C for one hour, after which the sections were washed three times in distilled water and once in 95% ethanol for 5 min. Next, sections were immersed in Nepthol Yellow S (sc215544; Santa Cruz Biotechnology, Heidelberg, Germany for 2 min at RT followed by 5 washing steps of 1 min each in distilled water. Subsequently, sections were immersed in Crystal Ponceau 6R (sc214779; Santa Cruz Biotechnology) for 10 min at RT followed by differentiating using 1% phosphotungstic acid (79690, Sigma-Aldrich) to remove all non-specific Crystal Ponceau staining. Finally, sections were immersed in Methyl Blue (M5528; Sigma-Aldrich) for 5 min at RT followed by washing in 1% acetic acid and rapid dehydration by transferring the sections through solutions of increasing ethanol concentration, until 100% ethanol was reached. Next, sections were immersed in a xylene-derivative Sub-X and mounted using Sub-X mounting medium (3801740, Leica).

DNA staining via Feulgen's reaction

For the Feulgen's reaction, sections were incubated with 5M hydrochloric acid for 50 min and subsequently with Schiff's Reagent (1079070001, Merck Chemicals, MA) for 1h at RT to

stain both intra- and extracellular DNA (pink). Next, sections were washed in 5% sodium bisulphite, 50 mM hydrochloric acid solution and rapidly dehydrated by transferring the sections through solutions of increasing ethanol concentration, until 100% ethanol was reached, followed by immersion in a xylene-derivative Sub-X (3803672E, Leica) and mounted using Sub-X mounting medium (3801740, Leica).

Immunohistochemistry

Thrombus sections were immunohistochemically examined for VWF, platelets (GPIbα) and leukocytes (CD45). Heat-induced antigen retrieval was performed for the leukocyte and the fibrin(ogen) staining using 0.3% sodium citrate (pH 6) at 95°C for 20 min, but not for the VWF and platelet staining. Before staining, thrombus sections were washed in Tris-buffered saline (TBS) containing 0.1% Tween 20, after which sections were blocked with 10% normal serum (swine or rabbit, dependent on the respective secondary antibody), 1 % bovine serum albumin (BSA) and 0.1 % Tween 20 in TBS for 1h. Next, sections were incubated overnight at 4°C with the primary antibody: rabbit anti-human VWF polyclonal antibody (1:1500, A008202-2, Dako, Glostrup, Denmark), mouse anti-human GPIbα monoclonal antibody (1:100, MA5-11642, Invitrogen, Waltham, MA), rabbit anti-human fibrin(ogen) polyclonal antibody (1:1000, A0080, Dako) or mouse anti-human CD45 monoclonal antibody (1:1000, 304002, Biolegend, San Diego, CA) in 1% BSA, 0.1% Tween 20 in TBS. After washing 3 times for 5 min in TBS 0.1% Tween 20 and blocking endogenous peroxidase activity using 0.3% hydrogen peroxide in TBS for 15 min at RT, a biotinylated secondary swine anti-rabbit IgG antibody (1:500, E043, Dako) or rabbit anti-mouse IgG antibody (1:200, E0413, Dako) in 1% BSA, 0.1% Tween 20 in TBS was added for 30 min at RT, followed by washing the sections 3 times for 5 min in 0.1% Tween 20 in TBS. To detect bound secondary antibody, an avidin-biotinylated HRP enzyme complex (Vectastain ABC kit, VEC.PK-6100, Vector Laboratories, Peterborough, UK) was added for 30 min at RT, followed by washing 3 times

for 5 min in 0.1% Tween 20 in TBS. VIP purple substrate (SK-4600, Vector Laboratories) and H2O2 were used, according to the manufacturer's instructions, to visualize the antigen of interest. Finally, the sections were incubated with a Methyl Green solution (H-3402, Vector Laboratories) for 8 min at 60°C as a counterstain to visualize all nuclei. Negative controls were obtained by omission of the primary antibody or by using isotype-matched primary antibodies (Supplementary Fig. 1A-D). Omission controls were performed by incubating a section with the diluent of the respective antibody (1% BSA, 0.1% Tween 20 in TBS) to control for aspecific signal from the detection system. Isotype controls were performed by incubating a section with the respective isotype of the primary antibody in 1% BSA, 0.1% Tween 20 in TBS (polyclonal rabbit IgG isotype control antibody (910801; Biolegend) for VWF or a monoclonal mouse IgG1κ isotype control antibody (401402, Biolegend)) to control for aspecific binding of the primary antibodies to the tissue.

Immunofluorescence staining

Thrombus sections were immunofluorescently examined for fibrin(ogen), VWF, platelets (GPIbα) and DNA. Heat-induced antigen retrieval was done using 0.3% sodium citrate (pH 6) at 95°C for 20 min. To perform multicolor immunofluorescent analysis, paraffin-embedded thrombus sections were then washed in TBS, blocked with 1% normal serum (donkey and/or goat, dependent on the combination of the respective secondary antibodies), 3% BSA and 0.1% Triton X-100 in TBS for 1h, and incubated overnight at 4°C with various combinations of primary antibodies: sheep anti-human VWF polyclonal antibody (1:250, ab11713, Abcam, Cambridge, UK), rabbit anti-human fibrin(ogen) polyclonal antibody (1:1000, A0080, Dako) and mouse anti-human GPIbα monoclonal antibody (1:100, MA5-11642, Invitrogen) in 1% normal serum (donkey and/or goat), 3% BSA and 0.1% Triton X-100 in TBS. The anti-human fibrin(ogen) antibody recognizes both fibrin and fibrinogen. After washing 3 times for 5 min in TBS, slides were incubated for 1h at RT with 1.5 µg/ml of the corresponding antibodies:

Alexa Fluor 488 donkey anti-rabbit IgG (A21206; Invitrogen), Alexa Fluor Plus 555 goat anti-mouse IgG (A32727, Invitrogen) or Alexa Fluor 647 donkey anti-sheep IgG (A21488, Invitrogen) in normal serum (donkey and/or goat), 3% BSA and 0.1% Triton X-100 in TBS. After washing three times five minutes in TBS, Prolong Gold Antifade Mountant with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) was used as a counterstain for DNA. Negative controls were achieved by omission of the primary antibody or by using isotype primary antibodies (Supplementary Fig. E,F). Omission controls were performed by incubating a section with the diluent of the respective antibody (1% BSA, 0.1% Tween 20 in TBS) to control for aspecific signal from the detection system. Isotype controls were performed by incubating a section with the respective isotype of the primary antibody in 1% BSA, 0.1% Tween 20 in TBS (polyclonal rabbit IgG isotype control antibody (910801; Biolegend) for VWF, a monoclonal mouse IgG1κ isotype control antibody (401402, Biolegend) and a polyclonal sheep IgG isotype control antibody (31243, Invitrogen)) to control for aspecific binding of the primary antibodies to the tissue. Combinations of the isotype primary antibodies are dependent on the used combinations of primary antibodies. Red blood cells were visualized using their inherent autofluorescence at a wavelength of 555 nm. Images from immunofluorescent stainings were acquired using an Axio Observer Z1 inverted fluorescent microscope (Zeiss, Carl Zeiss AG, Oberkochen, Germany) or a laser scanning confocal microscope (LSM710, Zeiss). Images were processed by Zen 2012 (blue edition, version 2.3, Zeiss) software.

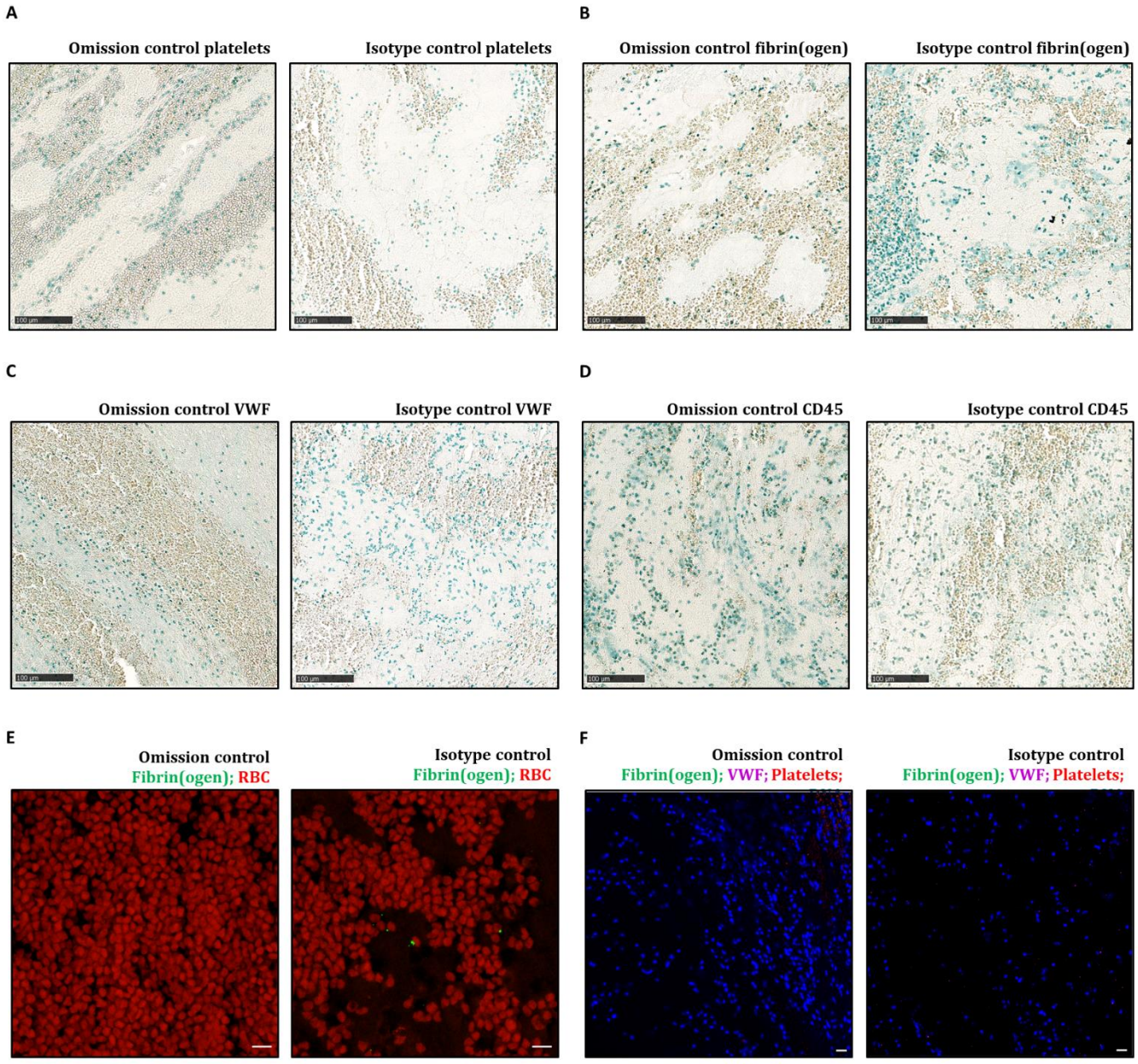
Quantification of RBC-rich and platelet-rich area

Quantification of RBC-rich and platelet-rich areas was performed on the MSB staining. Stained slides were digitized using a slide scanner (Nanozoomer SQ, Hamamatsu Photonics). Thrombus images were exported in a 5X resolution using NDP view2 Software (Hamamatsu,

Japan). Background surrounding the thrombus section was removed using Adobe Photoshop CS5 (San José, CA) to prevent false positive signal in the quantification. The MSB stainings were examined by color segmentation and planimetric analysis and the percentage of RBC-rich area (yellow) of the total section area was calculated using ImageJ software 1.49v (National Institutes of Health, Bethesda, MD; http://imagej.nig.gov/ij/). As we identified that the remaining areas (pink to red) are platelet-rich, the amount of platelet-rich area was calculated as 100% minus the determined % of RBC-rich area.

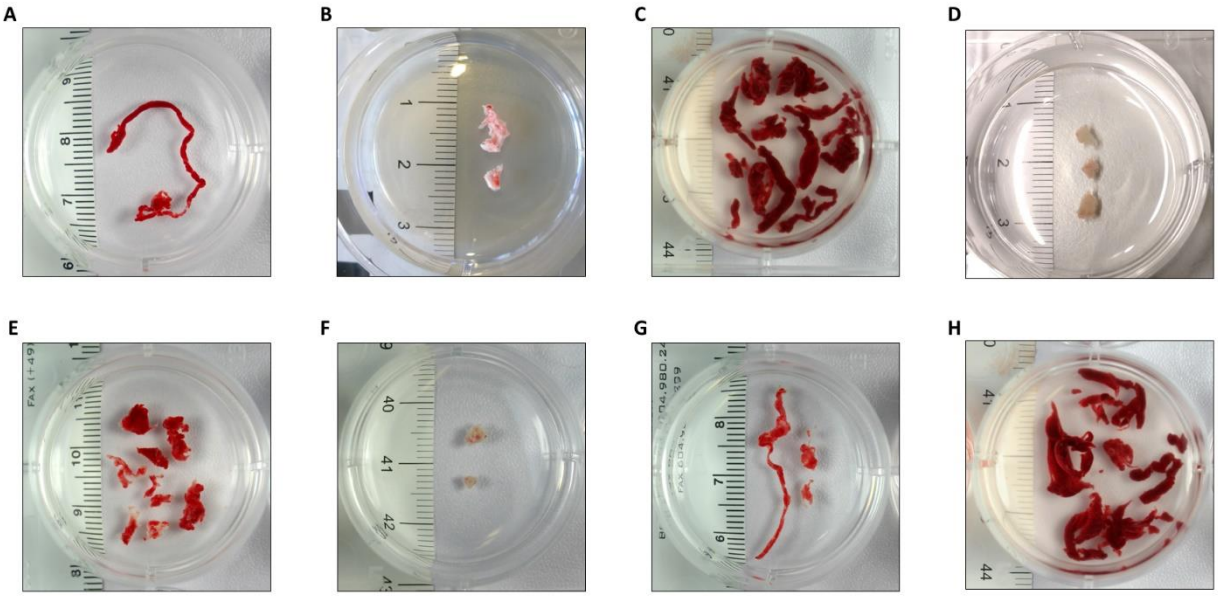
Supplemental Figures

Supplemental Figure 1



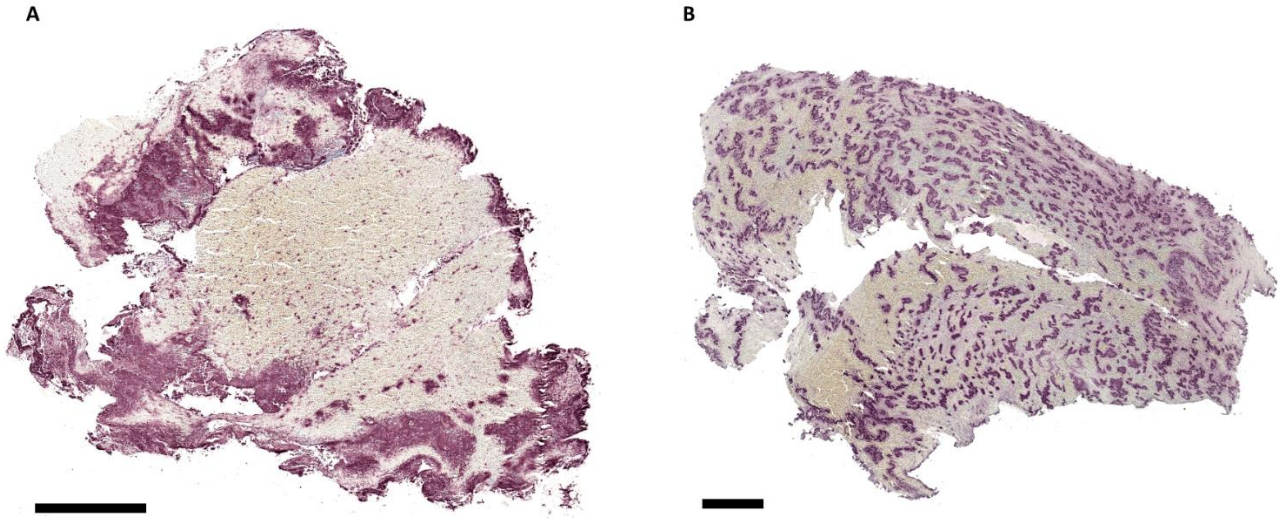
Supplemental Figure 1: Omission of primary antibody and isotype-matched negative controls. Omission and isotype-matched controls show that no positive signal (purple) is detected in the platelets (A), fibrin(ogen) (B), VWF (C) and CD45 staining (D). Omission and isotype-matched controls of immunofluorescent co-stainings show that no positive signal is detected of fibrin (E, F; green), VWF (F; violet) and platelets (F; red) but show the presence of autofluorescent RBC (E) and nuclei (F). Scale bars are 100 µm in panels A-D and 10 µm in panels E and F.

Supplemental Figure 2



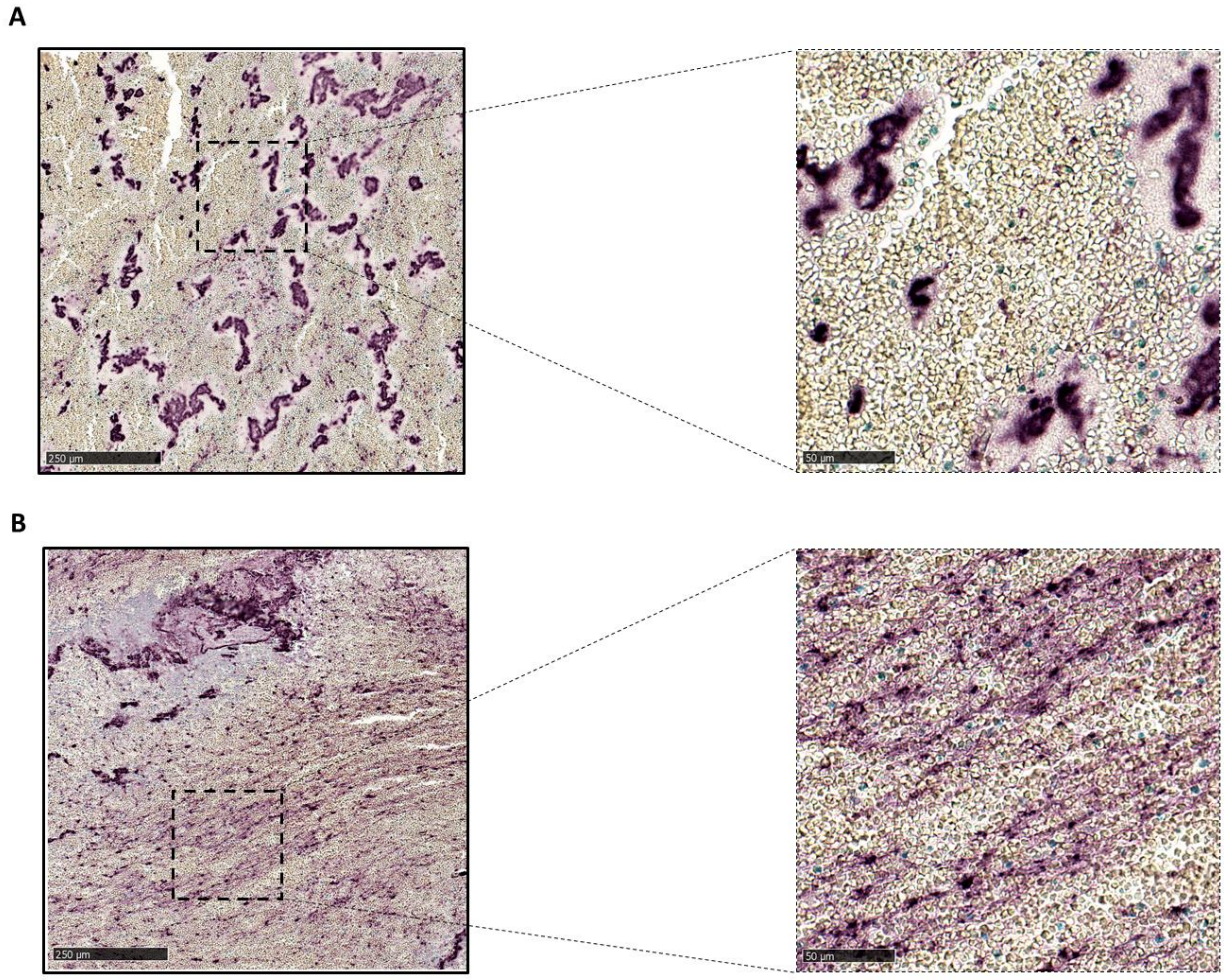
Supplemental Figure 2 Macroscopic pictures of fresh thrombi retrieved from stroke patients. After thrombectomy, thrombi are gently removed from the stent retriever. A picture of gross appearance of the removed thrombus material is taken. Stroke thrombi are heterogenous in size, shape, color and fragmentation. Eight thrombi (A-H), retrieved from eight patients are shown. Numbers on the scale are in cm.

Supplemental Figure 3



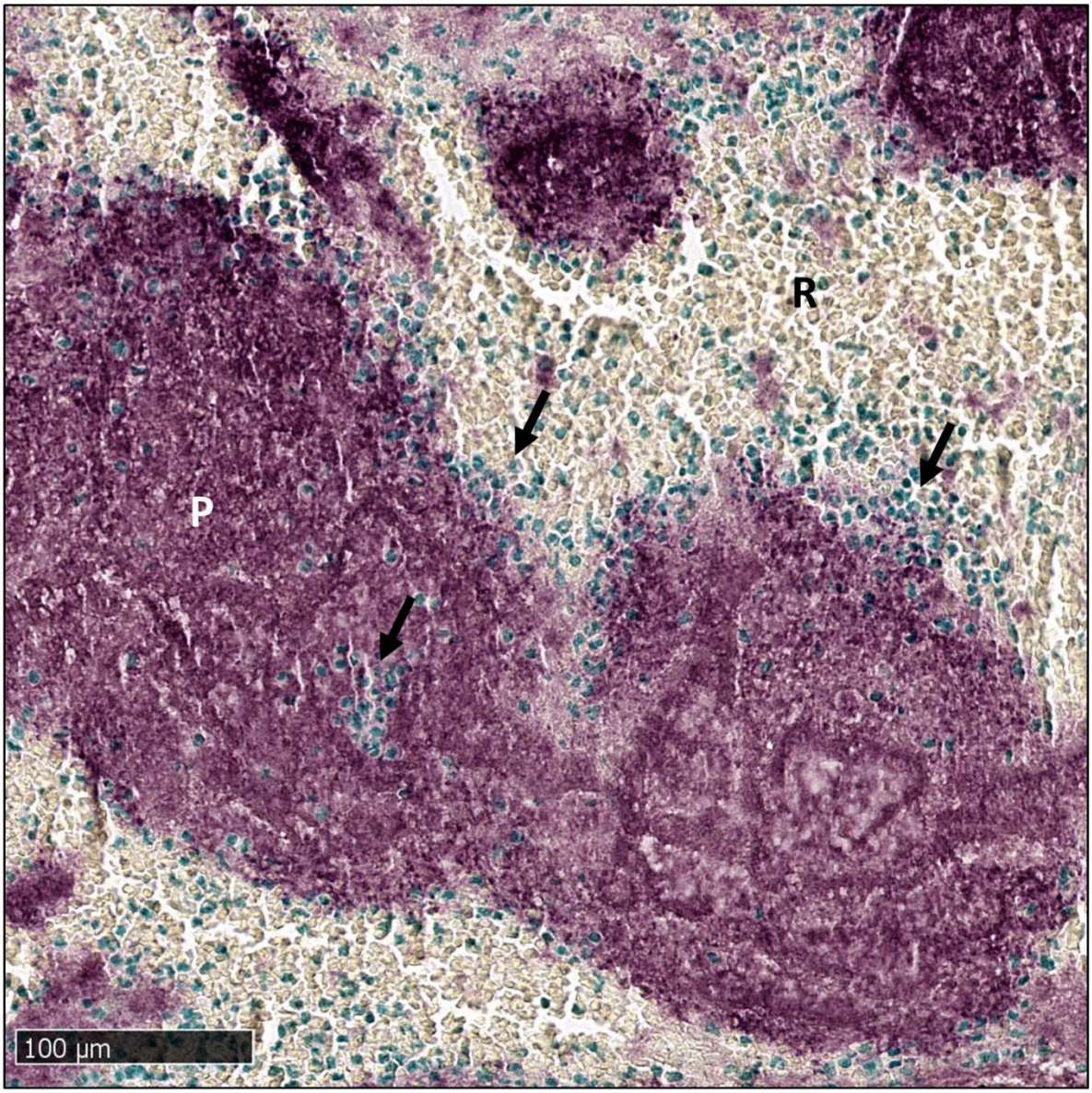
Supplemental Figure 3: Internal organization of platelet-rich areas and red blood cell-rich areas. Stroke thrombi were immunohistochemically analyzed for platelets (purple). (A) A thrombus containing a shell-like structure of platelet-rich material surrounding an RBC-rich core. (B) Platelet-rich areas are interspersed between RBC-rich areas without the clear presence of a platelet-shell. Scale = 500 µm.

Supplemental Figure 4



Supplemental Figure 4: VWF is rarely detected in RBC-rich areas. Stroke thrombi were immunohistochemically analyzed for VWF (purple). Unstained red blood cells have a yellowish color. (A) VWF is present in platelet-rich areas but not in RBC-rich areas in the majority of thrombi. Scale = 250 µm (left), 50 µm (right). (B) Only in few thrombi, VWF is seen in RBC-rich areas. Scale = 250 µm (left), 50 µm (right). Scale bar = 50 µm.

Supplemental Figure 5



Supplemental Figure 5: Leukocytes accumulate in and around platelet-rich areas. Stroke thrombi were immunohistochemically stained for platelets (purple) with a DNA counterstain (green). Leukocytes (black arrows) are seen on the boundary areas between platelet-rich and RBC-rich areas. Scale bar = 100µm. P = platelet-rich area, R = red blood cell-rich area.