Supplemental material

Altered fibrin clot structure and dysregulated fibrinolysis contributes to thrombosis risk in severe COVID-19

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Supplemental methods

Western blotting
Plasma (pre-diluted 1:40 with 0.9%NaCl) was separated on a SDS polyacrylamide gel, followed by electro-transfer to a PVDF membrane. After blocking with 5% non-fat dry milk in TBS buffer (25mM Tris pH 7.5, 150mM NaCl) supplemented with 0.1% Tween 20 (TBS-T), the membrane was incubated overnight at 4°C with a goat anti-FXII (cat. no.: 206-0056; Zytomed Systems, Berlin, Germany) or rabbit-anti high molecular weight kininogen (HK; cat. no.: ab35105; Abcam, Cambridge, UK) antibody. Next, the membrane was incubated with an appropriate peroxidase-labelled secondary antibody (all from Dako, Gostrup, Denmark). Final detection of proteins was performed using a Pierce™ ECL Western Blotting Substrate (Thermo-Fisher Scientific). As loading control, albumin was detected with a rabbit anti-albumin antibody (cat. no.: A001; Dako). Western blots were developed using a ChemiDoc™ Touch (Bio-Rad Laboratories, Inc., Hercules, CA), and densitometric analysis was conducted by the ImageLab™, Version 6.0.1 (Bio-Rad Laboratories).

Immunoassays
Factor XII levels in plasma were quantified by the Human FXII ELISA Kit from Abnova (Taipei, Taiwan). Plasma levels of plasminogen activator inhibitor-1 (PAI-1) and t-PA were measured using human ELISA Kits from Thermo- Fisher Scientific. Thrombin-activatable fibrinolysis inhibitor (TAFI) levels in plasma were quantified by the Human CPB2/TAFI ELISA Kit from LSBio (Seattle, WA). All measurements were performed according to manufacturer's instructions.

Immunostaining of clots generated in a purified system
Endogenous FXII was depleted from Influenza and Covid-19 plasma as described above. Following the addition of 40μg/ml FXII and 20mM CaCl₂, the plasma samples were incubated for 30min at 37°C to allow clot formation. Next, the samples were fixed with 4% paraformaldehyde and non-specific binding sites were blocked with 3% bovine serum albumin in PBS for 1h at room temperature. Afterwards, the clots were incubated with the rabbit anti-fibrinogen/fibrin antibody (cat. no.: A0080; Dako) overnight at 4°C. Following extensive washing with PBS, the clots were incubated with a secondary antibody labeled with Alexa Fluor™ 488 (Thermo-Fisher Scientific) for 1h
at room temperature. Finally, the clots were embedded in Vectashield Mounting Medium (Vector Laboratories Inc) and images were taken with the laser scanning confocal microscope (Leica TCSSP5, Leica, Wetzlar, Germany) using a Leica Plan Apo ×63/1.32 objective. ImageJ was used to determine fiber density, by counting the number of fibers crossing lines of 250µm placed in the image using the plug-in-grid. The observers were blinded to diagnosis.

**Immunostaining of human lung tissue samples**

Postmortem lung tissue specimens were fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Three micrometer sections were deparaffinized in xylene and rehydrated through graded ethanol washes. Antigen retrieval was performed by heating lung tissue section for 30min at 95°C in Tris-EDTA buffer (10mM Tris pH 9.0, 1mM EDTA). After antigen retrieval, the tissue sections were blocked with the blocking solution from the Zytochem-Plus AP Polymer Kit (Zytomed Systems) for 5min at room temperature and then incubated overnight at 4°C with the rabbit anti-fibrinogen/fibrin antibody (cat. no.: A0080; Dako, Gostrup, Denmark). Antigen detection was performed using a Zytochem-Plus AP Polymer Kit in accordance with the manufacturer's instruction. Stained lung tissue sections were digitalized using a Miramax-Slide-Scanner (Carl Zeiss, Wetzlar, Germany). ImageJ was used to determine fiber density, by counting the number of fibers crossing lines of 100µm placed in the image using the plug-in-grid, and to calculate fibrin positive vs total image area. The observers were blinded to diagnosis.

**Scanning electron microscopy**

Samples were fixed with 1.5% paraformaldehyde and 1.5% glutaraldehyde solution in 0.15M Hepes for 24h at room temperature. Next, samples were washed with 0.15M Hepes, post-fixed in 1% osmium tetroxide for 2h, washed in distilled water, dehydrated with graded ethanol washes and critical point dried by CO₂ treatment using a CPD030 critical point dryer (Evatec AG, Trübbach, Switzerland). Finally, samples were mounted with conductive adhesive tape and sputtered with gold. Images were taken with a Philips XL30 scanning electron microscope (Philips, Eindhoven, Netherlands). The SEM images were evaluated by two independent observers blinded to the study. They assigned pictures to the group according to the fibrin fiber density and fibrin fiber diameter.