Uncoupling of platelet granule release and integrin activation suggests GPIIb/IIIa as therapeutic target in COVID-19

Lukas J. Weiss,[1,2] Maria Drayss,[1,2] Georgi Manukjan,[1] Maximilian Zeitlhöfler,[1] Judith Kleiss,[1] Mathis Weigel,[1] Johannes Herrmann,[3] Kristina Mott,[1] Sarah Beck,[4] Philipp Burkard,[1] Thiên-Trí Lâm,[5] Karina Althaus,[6] Tamam Bakchoul,[6] Stefan Frantz,[2] Patrick Meybohm,[3] Bernhard Nieswandt,[1,4] Dirk Weismann*[2] and Harald Schulze*[1]

[1]Institute of Experimental Biomedicine, University Hospital Würzburg, Germany

[2]Department of Internal Medicine I, University Hospital Würzburg, Germany

[3]Department of Anaesthesiology, Intensive Care, Emergency and Pain Medicine University Hospital Würzburg, Germany

[4]Rudolf Virchow Center, University of Würzburg, Germany

[5]Institute for Hygiene and Microbiology, University of Würzburg, Germany

[6]Centre for Clinical Transfusion Medicine, University Hospital of Tübingen, Germany

* DW and HS contributed equally

Supplemental data

Correspondence:
Harald Schulze, PhD
University Hospital Würzburg
Institute for Experimental Biomedicine, Chair I,
Josef-Schneider-Str. 2/D15
97080 Würzburg
Germany
Phone: +49-931-201-48329
Fax: +49-931-201-648363
harald.schulze@uni-wuerzburg.de

Additional methods

Flow cytometry and data analysis

Flow cytometry and mepacrine assay were performed as described recently.[1,2] Geometric mean fluorescence intensity (GeoMFI) values were calculated using Flow-Jo V.10 (BD, Franklin Lakes, NJ, USA) and were normalized to allow a site by site comparison among separately recruited cohorts (Supplemental Fig. 2). Clustering analyses were calculated using the FlowSOM Vers.3.0.18 plugin for Flow-Jo after the event count has been adjusted using DownSample Vers. 3.3.1.[3]

Lumiaggregometry

Lumiaggregometry with platelet rich plasma (PRP) [platelet count >150/nl] was performed in a CHRONO-LOG device (Leiden, Netherlands) according to manufacturer's guidelines. Briefly, calibration was performed using a 2nM ATP standard. 50 µl of Chrono-lume luciferase reagent (both CHRONO-LOG) was added to 450 µl of PRP [final concentration: 166 U/ml] in prewarmed incubation wells and co-incubated for 3 minutes. Light transmission and the luminescence signal were recorded after agonist addition for six minutes at 37°C under stirring conditions (1000 rpm). Results were calculated using the Aggro-Link 8 software.

Point-of-care devices: PFA-200 and thrombelastometry

For patients with a platelet count of at least 150/nl, the in vitro-thrombus formation was assessed in 800 µl of whole blood, using the PFA-200 (Siemens Healthineers, Erlangen, Germany). The assay was performed according to manufacturer's guidelines using the collagen/ADP- and collagen/epinephrine-coated cartridges (Siemens Healthineers). We measured INTEM, EXTEM, or FIBTEM (Werfen, Barcelona, Spain) in whole blood by Rotational Thromboelastometry for 60 minutes.

Whole mount electron microscopy

For electron microscopy, carbon 200 mesh grids (Plano, Wetzlar, Germany) were coated with PRP for 60 seconds prior to dilution with distilled water. Grids were dried with filter paper and gently waved in the air to remove residual fluid.[4] Imaging was performed without additional fixation or staining using a JEOL-JEM 1400 microscope (JEOL Ltd., Tokyo, Japan). 20 representative images were acquired. δ-granule-number was determined by two blinded investigators. Data was analyzed using GraphPad Prism (Version 9.2.0, GraphPad Software, San Diego, CA USA). BioRender.com was used for figure illustration.

Flow Chamber

Cover slides were coated with 50 µg/ml Horm collagen (Takeda, Linz, Austria) for 12 hours at 37°C followed by 100 pM human recombinant tissue factor (Dade Innovin / Siemens Healthineers, Erlangen, Germany) for 1 h and blocked with 1% bovine serum albumin (BSA) for 1h. A parallel plate Maastricht flow chamber was rinsed with flow buffer (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, BSA 0.36%, glucose 0.1%, 2mM $MgCl_2$; pH 7.45). After preincubation for 5 minutes at 37°C with anti-GPIbβ-Alexa-Fluor 647 (p0p1, in house generated[5] and Alexa-Fluor 488-conjugated fibrinogen (Thermofisher Scientific Waltham MA, USA; final concentration: 3.125 ng/µL), citrate-anticoagulated whole blood was recalcified at the beginning of perfusion applying either arterial (γ=1000 s^{-1}; t=6 min) or venous shear rates (γ=200 s^{-1}, t=12 min). Images were acquired using a Leica DMI 6000 B fluorescence microscope (63x objective, NA 1.3, Leica DFC 360x camera, Wetzlar, Germany). After washing with Ca^{2+} enriched flow buffer (3.2 mM $MgCl_2$; 6.2 mM $CaCl_2$) for 1 minute (arterial shear) or 5 minutes (venous shear) five to ten representative images were obtained. Fixation for further

imaging was performed with 4% paraformaldehyde for 30 minutes. PBS washed slides were mounted with Fluoroshield (Sigma-Aldrich, St. Louis, MO, USA) and analyzed using a laser-scanning confocal microscope (Leica TCS SP8, Wetzlar, Germany) with a 63x oil objective, NA1.4 and LAS X software for image documentation. Image processing was performed with ImageJ (Version 2.0.0-rc-69/1.52p, NIH, MD, USA). For GPIIb/IIIa blockade whole blood was additionally incubated with different concentrations of eptifibatide (Sigma Aldrich, St Louis, MO, USA) or tirofiban (Sigma Aldrich, St Louis, MO, USA) for 20 minutes prior to perfusion. Brightfield images were assessed visually for thrombus morphology (morphological score), contraction (contraction score) and thickness (multilayer score) by two blinded investigators. Surface area coverage (% SAC) of fibrin(ogen) and platelets and thrombus intensity were measured in immunofluorescence images using semi-automated ImageJ / Fiji-scripts.[6]

Statistical analyses

The statistical tests to address significance are specified in the respective figure legends.

References

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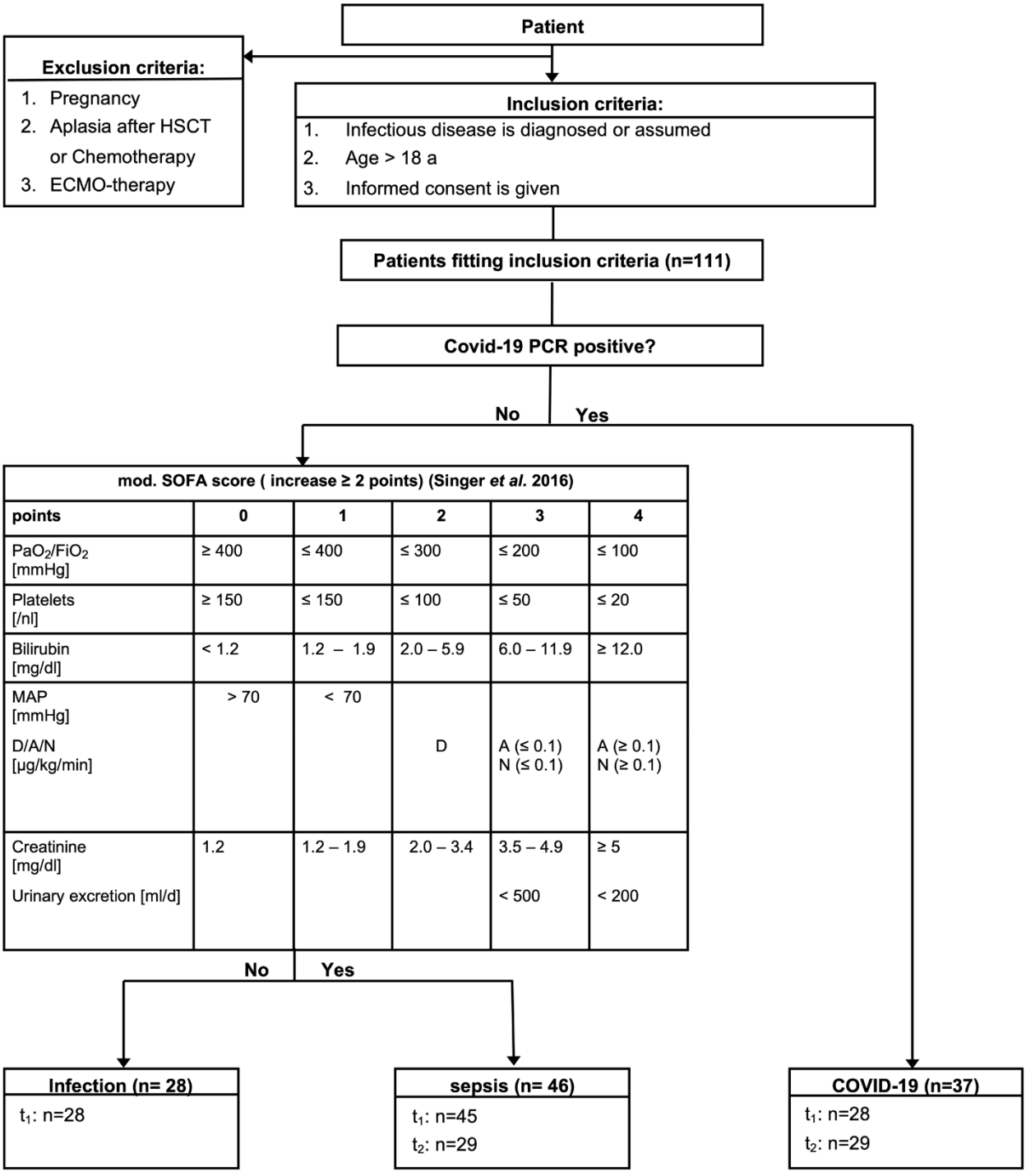
Supplementary Table 1: Blood parameters from healthy controls

	Healthy ctrl	
Anticoagulant	**Citrate (n=35) 61 measurements**	**EDTA (n=22) 25 measurements**
Age, median (IQR)	25 (23-30)	
Laboratory values, median (IQR)		
PLT	203 (185-246)	266 (224-296)
RBC	4.42 (4.10-4.64)	4.80 (4.45-5.14)
WBC	5.6 (4.6-6.2)	6.1 (4.9-6.7)
Hb	13 (12.2-13.7)	14.1 (13.2-14.8)
Hct	38 (35-40)	42 (39-43)

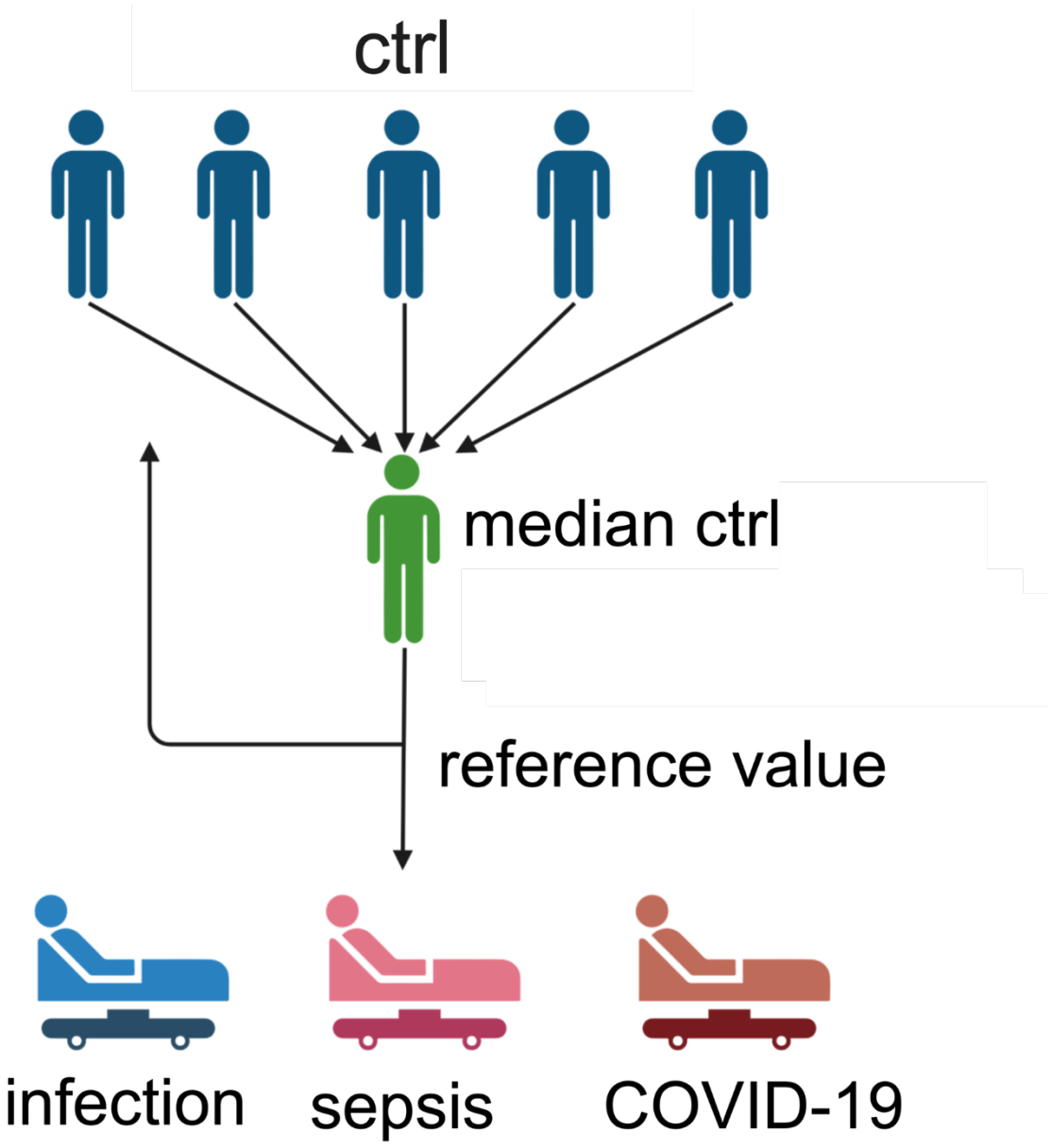
Abbreviations:
IQR: Interquartile range
PLT: Platelet
RBC: red blood cell count
WBC: White blood cell count
Hb: Hemoglobin
Hct: Hematocrit

Of 35 healthy donors, 61 measurements have been performed at different time points. Blood cell counts were determined with a Sysmex KX21N for all measurements (middle column). For 22 out of these 35 controls additional EDTA-based blood cell counts were available with a total of 25 measurements (right column). The data from this column reflect the patient data compiled in Table 1.

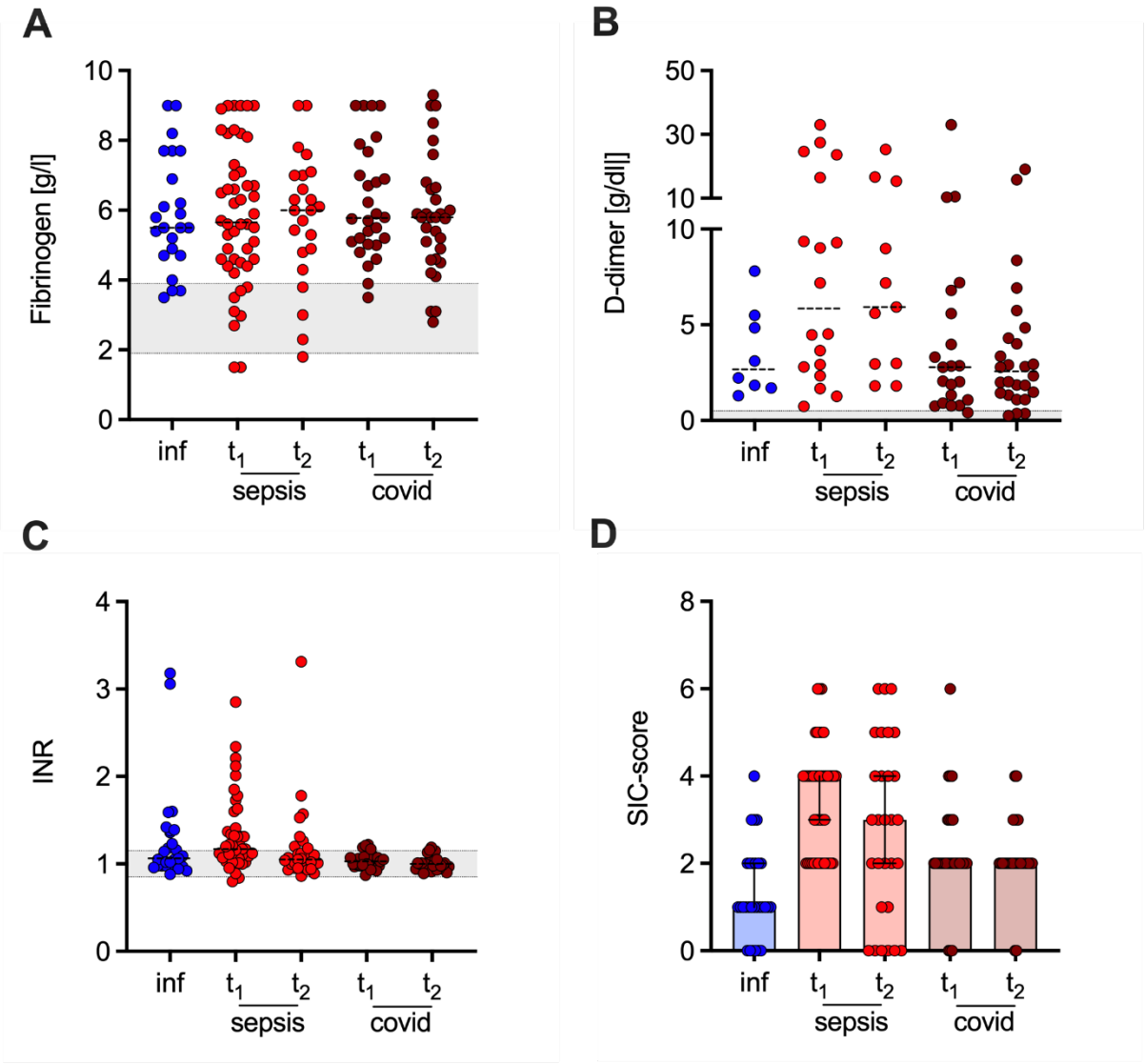


HSCT: Hematopoietic stem cell transplantation
MAP: Mean arterial pressure
D/A/N: Dobutamine, adrenaline, nordadrenaline

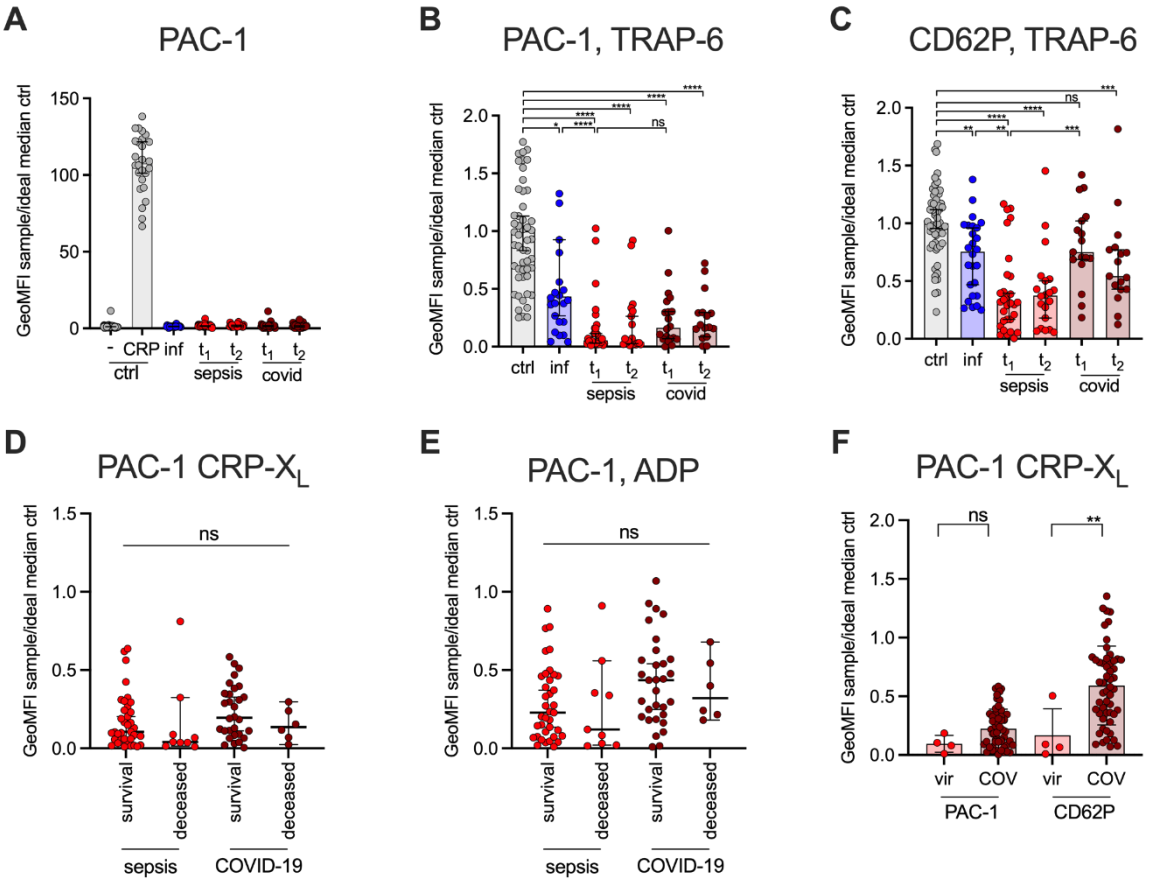
Supplemental Figure 1: Inclusion-algorithm for patients



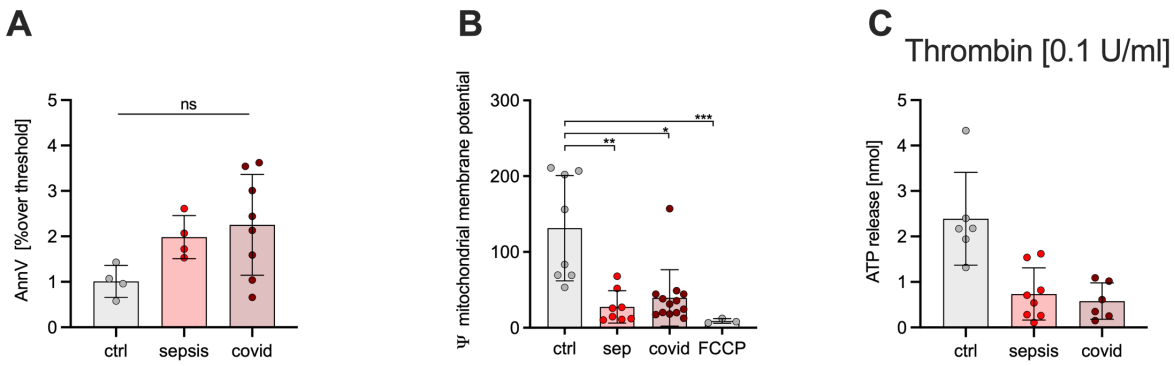
Supplemental Figure 2: Normalization scheme: median healthy control GeoMFI values were defined and used as reference for patient cohorts.



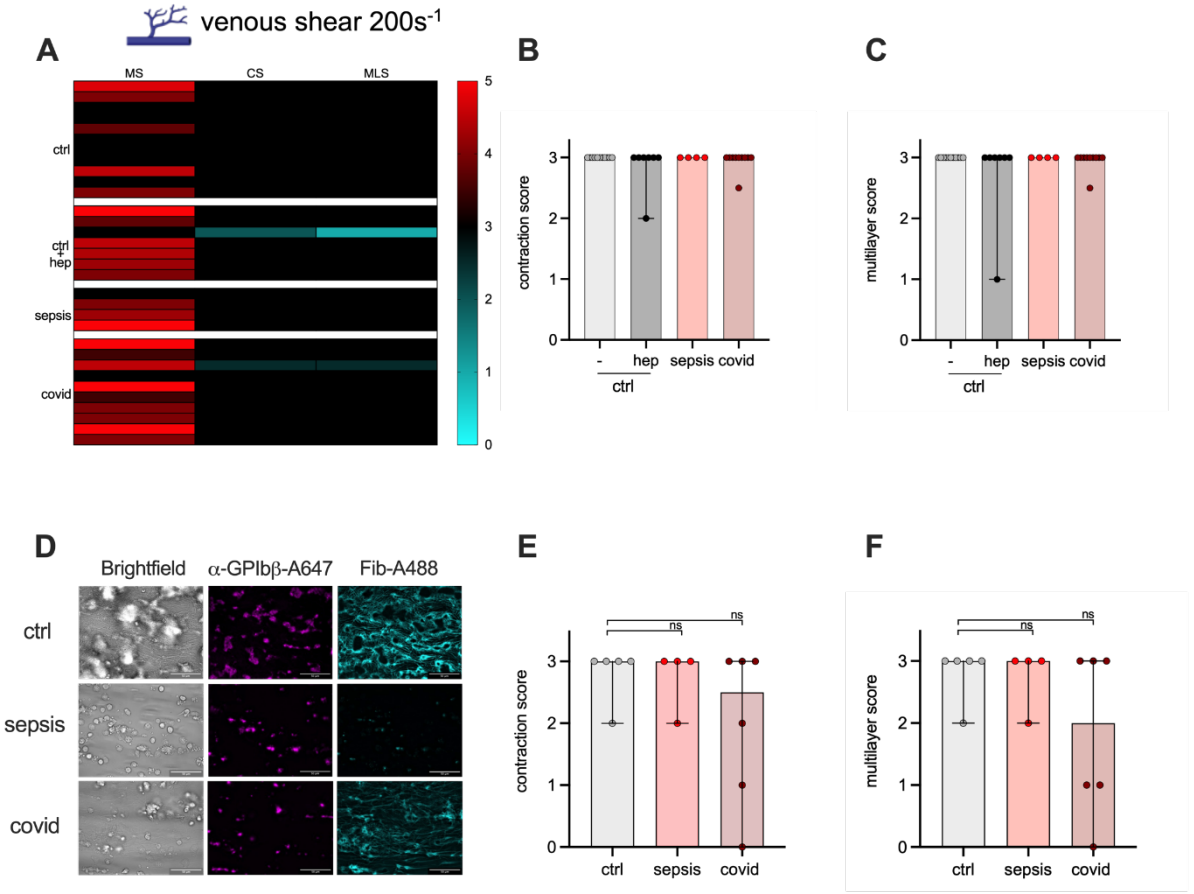
Supplemental Figure 3: Acute phase reaction and altered coagulation
(A) Fibrinogen-level, **(B)** D-dimers, **(C)** international normalized ratio and **(D)** sepsis-induced coagulopathy (SIC) score of patients with infection (inf), sepsis or COVID-19 are displayed at: t_1: hospital/ICU admission day; t_2: day 4 to 7. Reference ranges are indicated by dashed lines. Graphs **A-C** display median and **D** median ± 95% CI.



Supplemental Figure 4: Hyporeactive platelets in sepsis and COVID-19
Characteristics of healthy controls (ctrl), patients with infection (inf), sepsis or COVID-19 are displayed at: t_1: hospital/ICU admission day; t_2: day 4 to 7. **(A)** Normalized GPIIb/IIIa activation under resting conditions and upon stimulation of ctrls with CRP-X_L (CRP). **(B)** GPIIb/IIIa activation and **(C)** CD62P surface exposition upon TRAP-6 stimulation [5 µM] were determined by flow cytometry. GPIIb/IIIa activation upon stimulation with **(D)** CRP-X_L or **(E)** ADP of last available measurement of surviving and deceasing patients. **(F)** GPIIb/IIIa activation and CD62P surface exposition upon stimulation with CRP-X_L of patients suffering from viral infection in the sepsis cohort vs. COVID-19 patients. All graphs show median ± 95%CI. **(B-C)** Differences were analyzed using Kruskal-Wallis test or **(D-F)** Mann-Whitney test ns: nonsignificant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

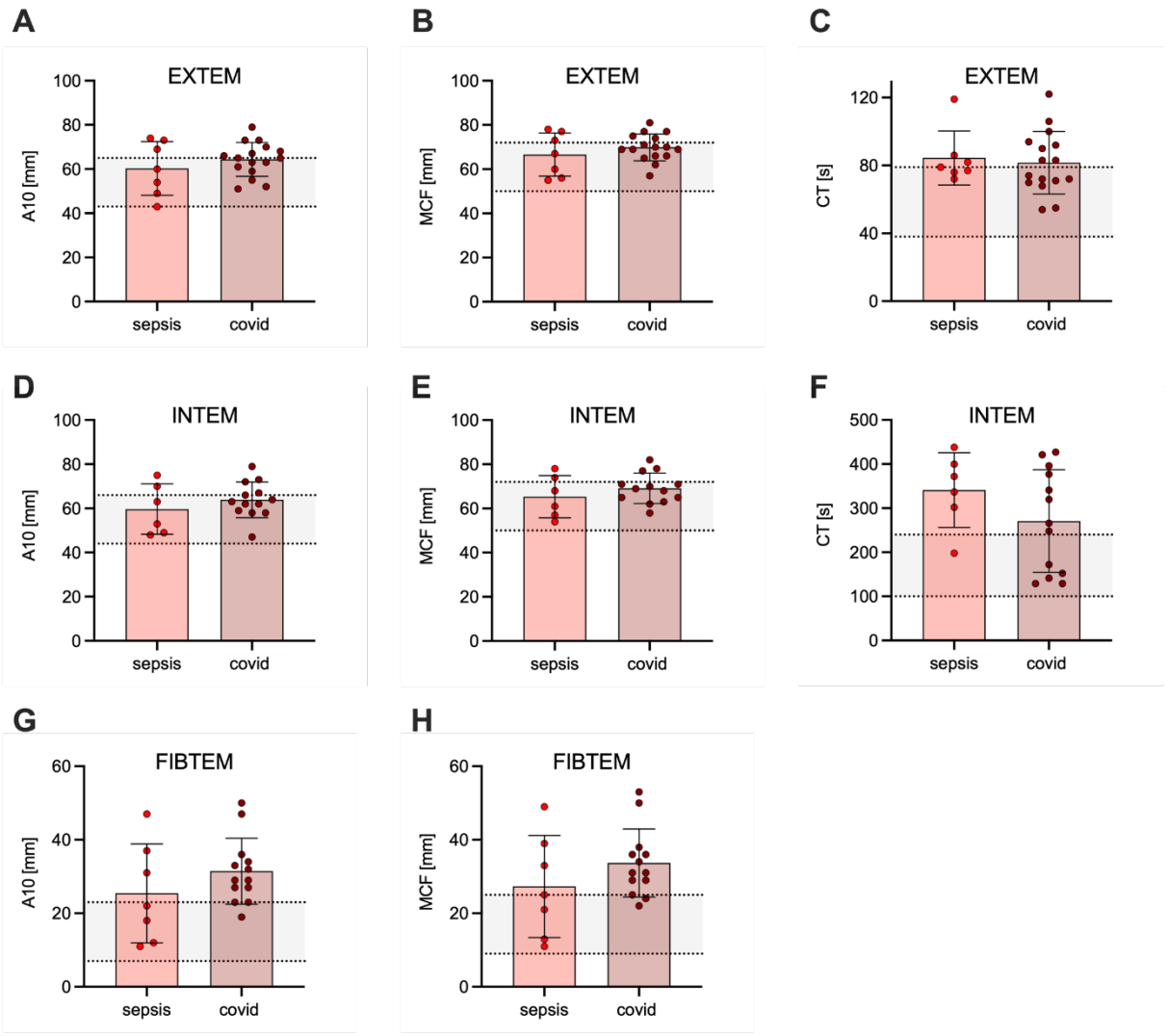


Supplemental Figure 5: Procoagulant platelets in sepsis and COVID-19
Characteristics of healthy controls (ctrl), patients with infection (inf), sepsis or COVID-19 are displayed at: t_1: hospital/ICU admission day; t_2: day 4 to 7. **(A)** Phosphatidylserine surface exposition was measured by Annexin V binding. **(B)** Mitochondrial membrane potential was assessed using TMRE **(C)** ATP release was measured by lumiaggregometry in PRP upon thrombin stimulation [0.1 U/ml] (platelet count >150/nl). All graphs show median ± 95% CI. Differences were analyzed using the Kruskal-Wallis test. ns: nonsignificant; *p<0.05; **p<0.01; ***p<0.001.

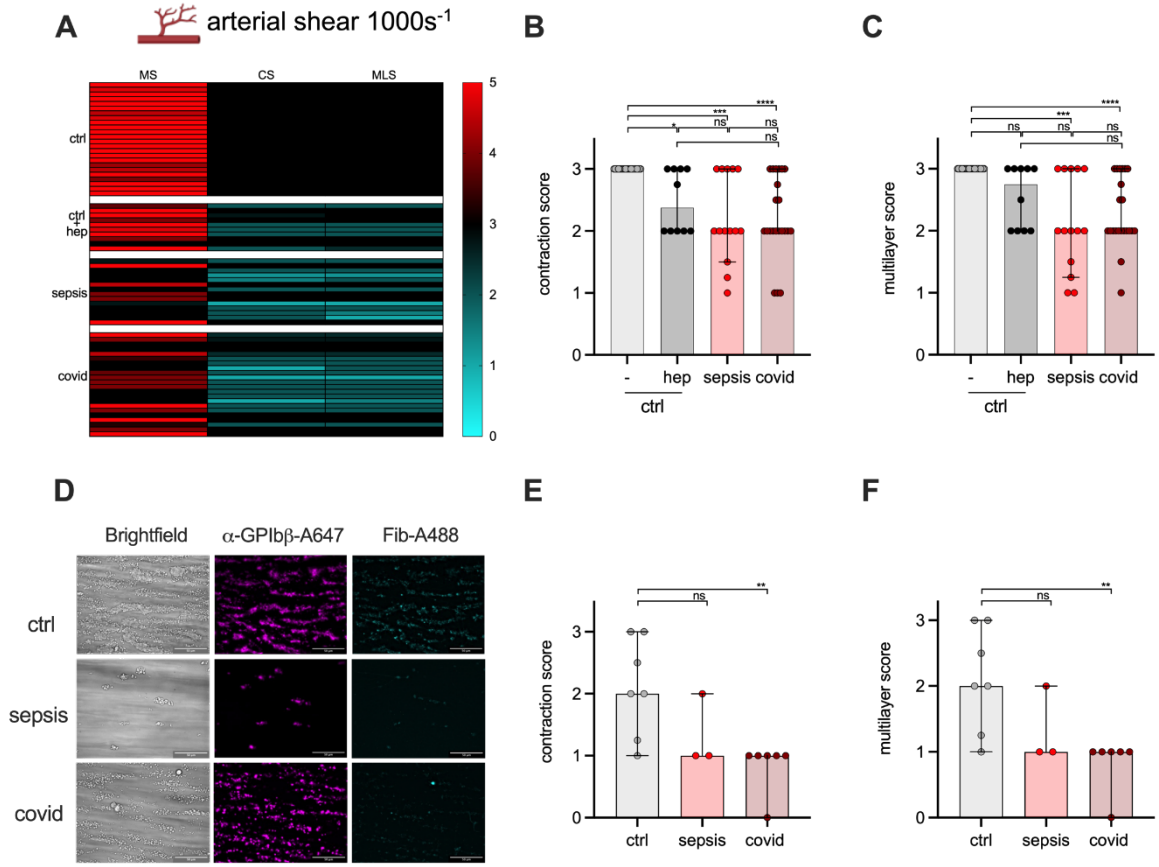


Supplemental Figure 6: **Robust thrombus formation in COVID-19 and sepsis patients under venous shear**

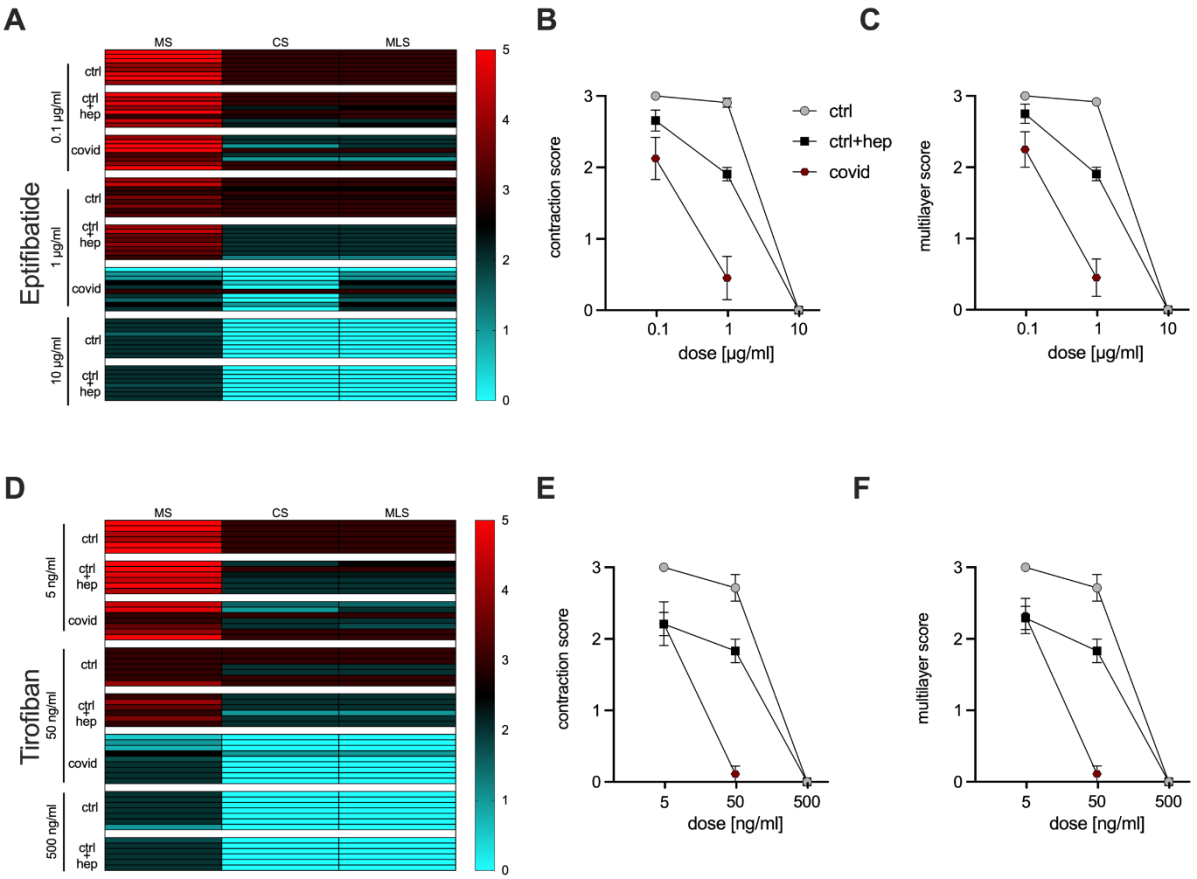
Thrombus formation was assessed under venous shear (200 s^{-1}) in recalcified whole blood on **(A-C)** collagen and tissue factor or **(D-F)** only collagen coated slides. (**B,E**) Contraction score and **(C,F)** multilayer score calculated in a two investigator blinded approach. **(A)** Quantitative imaging of morphological score, contraction score and multilayer score in a heat map for each individual ctrl and patient. Representative figures are displayed in **D. B, C, E, F** show median ± 95%CI. Differences were analyzed using Kruskal-Wallis test. ns: nonsignificant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.



Supplemental Figure 7: **Hypercoagulability during critical infection**
(A-C) EXTEM, **(D-F)** INTEM and **(G-H)** FIBTEM were determined by Rotational Thromboelastometry analysis in patients assessing **(A,D,G)** amplitude at 10 mins (A10) **(B,E,H)** maximum clot firmness (MCF) or **(C,F)** clotting time (CT). Reference ranges are indicated by dashed lines.

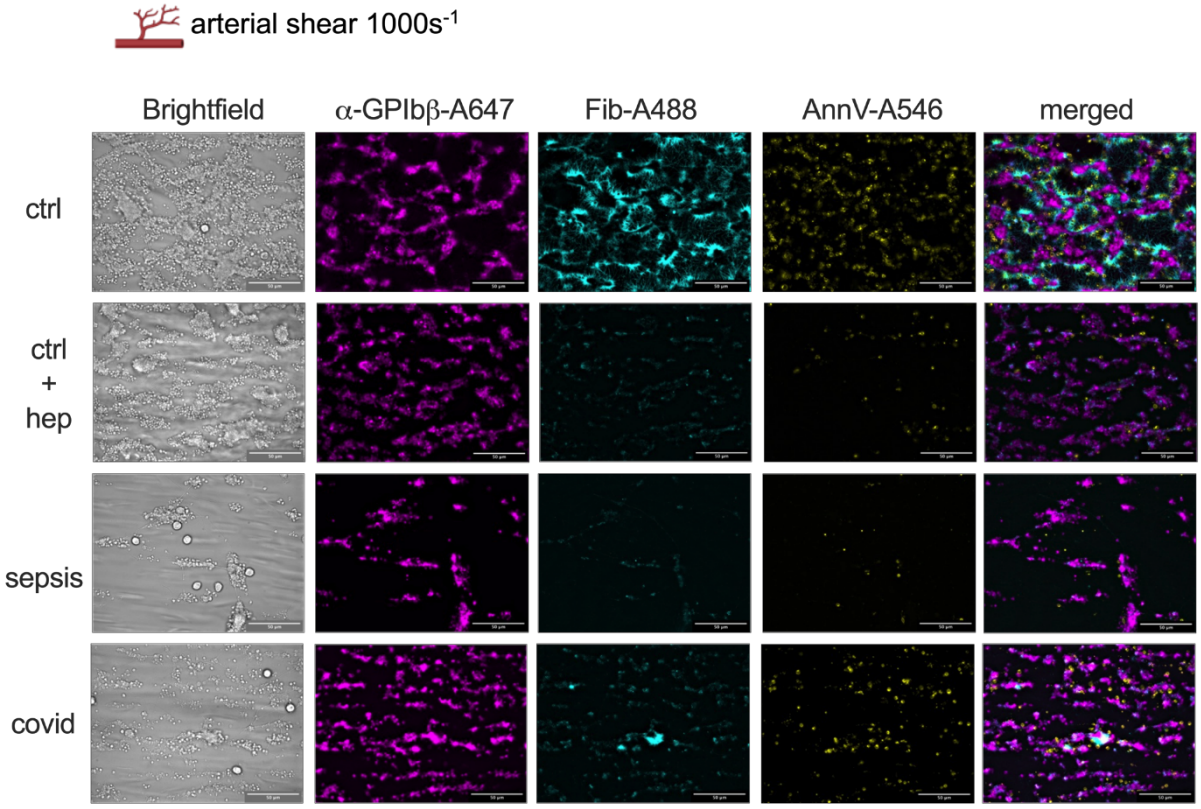


Supplemental Figure 8: **Robust thrombus formation in COVID-19**
Thrombus formation was assessed under arterial shear (200 s^{-1}) in recalcified whole blood on **(A-C)** collagen and tissue factor or **(D-F)** only collagen coated slides. (**B,E**) Contraction score and **(C,F)** multilayer score calculated in a two investigator blinded approach. **(A)** Quantitative imaging of morphological score, contraction score and multilayer score in a heat map for each individual ctrl and patient. Representative figures are displayed in **D. B, C, E, F** show median ± 95%CI. Differences were analyzed using Kruskal-Wallis test. ns: nonsignificant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

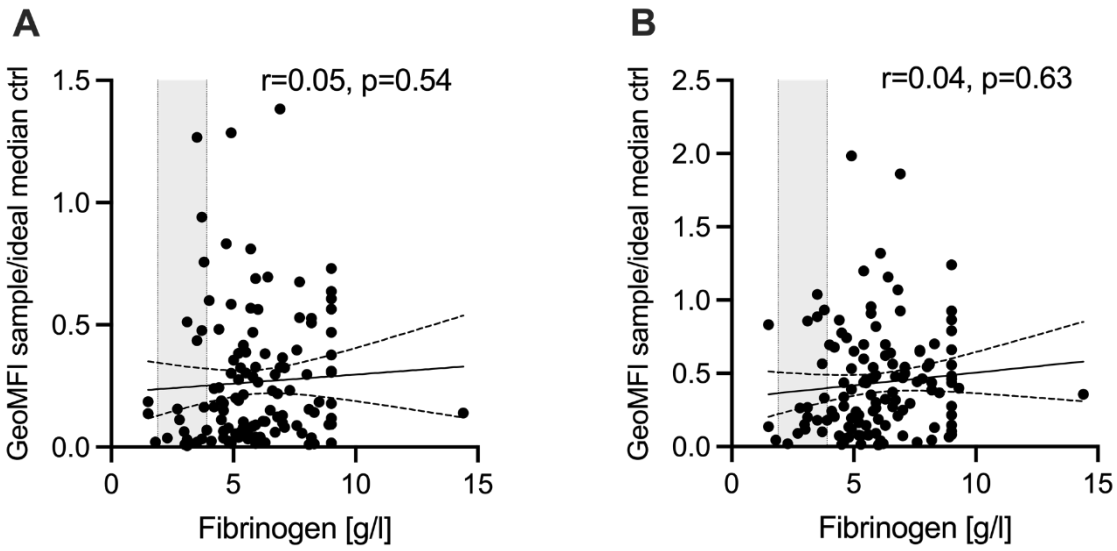


Supplemental Figure 9: **Low dose GPIIb/IIIa blockade prevents thrombus formation in COVID-19**

Thrombus formation on collagen/TF was assessed under arterial shear (1000 s^{-1}) in recalcified whole blood after preincubation with **(A-C)** eptifibatide or **(D-F)** tirofiban at the indicated concentrations. **(B,E**) Contraction score and **(C,F)** multilayer score were calculated in a two investigator blinded approach. **(A,D)** Quantitative imaging of morphological score, contraction score and multilayer score in a heat map for each individual ctrl and patient. **B, C, E, F** show mean ± SEM. Differences were analyzed using Kruskal-Wallis test. ns: nonsignificant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.



Supplemental Figure 10: **Annexin V-positive events in the coagulation flow chamber model**
Thrombus formation on collagen/TF was assessed under arterial shear (1000 s^{-1}) in recalcified whole blood. Samples were stained for platelets (α-GPIbβ-Alexa647: magenta), fibrin(ogen) (Alexa488: cyan) and phosphatidylserine (Annexin V-Alexa546: yellow [0.5 µg/ml]).



Supplemental Figure 11: **No correlation of plasma fibrinogen levels with PAC-1 binding to platelets.** Plasma fibrinogen levels vs. GPIIb/IIIa activation (PAC-1-binding) upon stimulation with **(A)** CRP-X_L or **(B)** ADP.