Supplemental information

Heparan sulfate mimetic fucoidan restores the endothelial glycocalyx and protects against dysfunction induced by serum of COVID-19 patients on ICU

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Supplementary Material and methods

Circulating markers of endothelial dysfunction and glycocalyx shedding

Plasma samples were thawed at room temperature. All plasma analytes were measured with immunoassays in duplicate according to manufacturer's recommendation. Samples were diluted in diluent reagent according to manufacturer's protocol. Analytes measured include angiopoietin 2 (Ang-2, DANG20, R&D Systems, Abingdon, UK), soluble thrombomodulin (sTM, M850720096, Diaclone, Besançon, France) and soluble syndecan-1 (sSDC1, DY2780, R&D Systems). For angiopoietin 2 and soluble thrombomodulin, commercial control samples were included in each plate to confirm the amount of protein level.

***In Vitro* Culture Experiments**

Primary human pulmonary microvascular endothelial cells (HPMECs) were purchased from PromoCell (Lot no. 455Z003, Heidelberg, Germany) and primary human glomerular microvascular ECs (GEnCs) were purchased from Cell Systems (ACBRI-128; Kirkland, WA, USA) and were both cultured in endothelial growth medium (basal medium MV, C-22220, PromoCell) supplemented with C-39220 (PromoCell) and 1% antibiotics (penicillin/streptomycin, 15070063, Gibco, Paisley, UK) at 37°C and 5% CO_2. For each cell line, passage 5 were used for experiments.

Endothelial barrier function assay

Endothelial barrier function analysis was performed with impedance-based cell monitoring using the electric cell-substrate impedance sensing system (ECIS Zθ, Applied Biophysics, New York, USA). ECIS plates (96W20idf PET, Applied Biophysics) were pre-treated with 10 mM L-cysteine and coated with 1% gelatine (for GEnCs) or without 1% gelatine (for HPMECs). HPMECs (p5) were seeded at a concentration of 4.5×10^5 cells/well and for GEnCs (p5), the concentration was 3×10^5 cells/well. Medium was refreshed after 24 hours. Initial baseline resistance was measured for 2 hours before endothelial cells were seeded into the plate. Multiple frequency/time mode was used for the real-time assessment of the barrier function. Additionally, the real-time resistance was generated every 350 seconds. Once the stable monolayer was formed, endothelial cells were incubated with 10% serum (healthy (n=12), COVID-19 non-ICU (n=8) and ICU (n=26)) and measured for 20 hours. Afterwards, modelling data Rb which represents barrier function could be generated.

In additional experiments, HPMECs were exposed to 10% healthy serum (n=12), COVID-19 ICU serum (n=26) with or without fucoidan (10 µg/mL, gift from MicroVascular Health Solutions LLC, Alpine, UT, USA) and measured on the ECIS machine for 20 hours. The natural fucoidan provided (Iso 9000 and GMP certified from Omnipharm, S.A.S, Chambéry, France) was extracted from *Laminaria japonica* as a powder of 91.20% purity and further tested, for instance, on the presence of heavy metals (arsenic, lead, cadmium, mercury) or microbiology parameters (European Pharmacopoeia VIII, Ed 2,6,12: total plate count, yeast, mold, *E. coli, Salmonella spp.*). A 50x times stock solution was prepared by dissolving the appropriate amount of powder in milliQ water and passed through a 0.22µm filter before use. Cell culturing supernatant and 10% serum in no FCS medium were collected and centrifuged for VWF ELISA assay.

RNA isolation and RT-PCR

Cells were harvested in TRIzol and total RNA was isolated using RNeasy Mini Kit (74106, Qiagen, Venlo, The Netherlands) according to its protocol. cDNA synthesis was transcribed using GoScript™ Reverse Transcriptase kit (A5001, Promega, Leiden, The Netherlands). RT-PCR analysis was conducted using SYBR Select Master Mix (4472908, Applied Biosystems, Landsmeer, The Netherlands) and specific primers as indicated in supplementary table s2. Gene expression was normalized to GAPDH of 5 separate experiments.

Immunoblotting analysis

Western blots were performed from protein extracts of HPMECs. Cells were washed once with cold PBS and lysed in lysis buffer mentioned in our previous study[1]. After centrifugation of the samples at 12,000 rpm for 15 min at 4°C, supernatant was collected and protein concentration was measured using the Pierce BCA Protein Assay Kit (23255, ThermoFisher, Landsmeer, The Netherlands). Equal amounts of protein were denatured using DTT and incubated at 95°C for 10 minutes. 10% Mini-PROTEAN® TGX™ Protein Gels (4561031, Bio-Rad Laboratories, Veenendaal, The Netherlands) were used for protein size separation and proteins were transferred to PVDF membranes (1704156, Bio-Rad) using the Trans-Blot Turbo system. Afterwards, the membrane was blocked in 5% milk in PBST at room temperature for 1 hour. Primary antibody rabbit anti-human ICAM1 (4915, Cell Signalling Technology), rabbit anti-human total NF-κB p65 (8242, Cell Signalling Technology), rabbit anti-human phosphor-NF-κB p65 (Ser536) (3033, Cell Signalling Technology) and mouse anti-human

GAPDH (MA5-15738, ThermoFisher) were incubated overnight at 4°C. Incubation with a secondary HRP-conjugated antibody (P0447 and P0448, Dako, Amstelveen, The Netherlands) and visualization using Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (NEL103001EA, PerkinElmer, Groningen, The Netherlands). Intensity of the bands were analysed using ImageJ software. Relative intensity was determined by levels of GAPDH.

Immunofluorescence of cultured cells

HPMECs (p5) were cultured in 8-well chamber slides (ibiTreat, µ-Slide 8 Well). Medium was refreshed after 24 hours to remove non-attached cells. When HPMECs formed a confluent monolayer after 48 hours, they were incubated with 10% pooled serum (healthy control (pooled n = 12), non-ICU (pooled n = 8) and ICU (pooled n = 26) in no FCS medium for 24 hours. Next, cells were fixed with 4% PFA and 0.2% Triton-X100 in HBSS or 4% PFA in HBSS (for HS and LEA staining) for 10 minutes at room temperature, washed twice with 1% BSA in HBSS, and blocked with 3% goat serum in HBSS for 1 hour at room temperature. Then, cells were incubated with FITC labelled *Lycopersicon esculentum* (LEA-FITC, L0401, Sigma, Houten, The Netherlands), primary monoclonal Mouse Anti-Human VE cadherin (55-7H1; BD Biosciences), monoclonal Mouse Anti-heparan sulfate (10E4, 370255-1, AMSBIO, Abingdon, UK) and monoclonal Mouse Anti-TM (MA5-11454, ThermoFisher) at 4°C overnight, followed by an appropriate secondary antibody and phalloidin-TRITC (VE-cadherin samples) for 1 hour, all in blocking buffer. Cells were subsequently washed and incubated with Hoechst 33528 (1/1,000) for 10 minutes at room temperature.

Cells were imaged using a LEICA SP8 WLL confocal microscope (Leica , Rijswijk, the Netherlands) to create image stacks. Fluorescent images were analysed using Image J software. To quantify the total area of VE-cadherin staining, all VE-cadherin derived fluorescent signal below a threshold was included in a mask. The value for the threshold intensity was kept the same for each field and then the positive area of VE-cadherin is quantified as adhesion junction area. 10 fields per experiment were imaged for every condition for further analysis To quantify the glycocalyx coverage, endothelial nuclear region was selected and the thickness of the glycocalyx was quantified by calculating the distance from the half-maximum signal of the nuclear staining at the luminal side to the half-maximum signal at the luminal end of the staining in the z-direction. Fluorescence intensity was calculated based on the average fluorescence intensity from the half-maximum signal of the nuclear staining at the luminal side to the half-maximum signal at the luminal end of the staining in the z-

direction. Surface amount of glycocalyx was presented as mean fluorescence intensity (Mean FL) times luminal thickness.

FX activation by extrinsic tenase complex (TF-FVIIa)

Primary human pulmonary microvascular endothelial cells at passage 5 were seeded into 96 wells plates at a concentration of $2.5x10^4$ cells/well. Medium was refreshed after 24 hours and after 48 hours, HPMECs were incubated with 10% control (n=12), COVID-19 non-ICU (n=8) and ICU serum (n=26, with or without presence of fucoidan) in no FCS medium for 24 hours. Cell culture supernatants were collected and centrifuged for ELISA assays. Afterwards, cells were washed once with prewarmed HBSS with Ca^{2+}. Factor VIIa (80 µL, 10 nM, HCVIIA-0031, Haematologic Technologies, Huissen, The Netherlands) was added and incubated for 15 minutes at 37°C and 5%CO_2. Then the reaction system was initiated by adding factor X (80 µL, 400 nM, HCX-0050, Haematologic Technologies). The plate was kept in incubator to keep the reaction continuous and 2 time points were selected (1 hour and 2 hours) to detect the production of active factor X. Aliquots were taken and quenched in HBS supplemented with 50mM EDTA to stop the reaction. The amidolytic activity of each sample was determined by SpecXa conversion (250 µM), measuring the absorbance at 405 nm and the initial rates of chromogenic substrate hydrolysis were converted to nanomolar of product by reference to a corresponding FXa standard curve.

Thrombin generation Assay

Primary human pulmonary microvascular endothelial cells at passage 5 were seeded into 96 wells plates at a concentration of $2.5x10^4$ cells/well. Medium was refreshed after 24 hours and after 48 hours, HPMECs were incubated with 10% healthy (n=12), COVID-19 non-ICU (n=8) and ICU serum (n=26, with or without presence of fucoidan) in no FCS medium for 24 hours. Warm HBSS without Ca^{2+} was used to wash the cells once. Thrombin generation curves were obtained by supplementing normal pooled plasma. Thrombin formation was initiated by adding substrate buffer (FluCa-kit, 86197, Thrombinoscope BV, Leiden, The Netherlands) to the plasma. The final reaction volume was 120 µL, of which 80 µL was plasma, 20 µL was HBSS/calibrator and 20 µL was substrate. Thrombin formation was determined every 30 s for 120 min and corrected for the calibrator using Thrombinoscope software.

Markers of endothelial dysfunction and glycocalyx shedding in cell cultured supernatant

Supernatants collected from experiments mentioned above and stored at -80°C were measured with immunoassays in duplicate according to manufacturer's recommendation. Analytes measured include angiopoietin 2 (Ang-2, DANG20, R&D Systems), soluble thrombomodulin (TM, M850720096, Diaclone), IL-6 (M9316, Sanquin, Amsterdam, The Netherlands) and VWF (A0082, Dako). Samples were diluted 1:100, 1:5, 1:10 and 1:200 respectively in diluent reagent. For angiopoietin 2 and soluble thrombomodulin, commercial control samples were included in each plate to confirm the amount of protein level.

Public database mining

To further study the role of thrombomodulin in endothelial cells from different vascular beds, datasets from EC atlas (https://endotheliomics.shinyapps.io/ec_atlas/ [2]) and NCBI Gene expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) were downloaded. We then determined Thbd expression in mice ECs from 11 organs. For human ECs, 2 datasets (GSE43475 [3] and GSE21212 [4]) were downloaded and processed with RMA background correction, log2 transformation and normalization in R. For annotation, we chose the gene with the highest expression as the gene symbol and annotated it via different annotation packages. Then THBD expression was detected in different EC lines.

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Supplementary table S1 Demographic, clinical, and outcome data of patients in BEAT-COVID cohort

	Hospitalized (n = 32)	ICU (n =26)	Non-ICU (n = 6)	Healthy control (n = 12)
Age - years median (IQR)	61 (57-70)	62 (58-71)	56 (47-65)	60 (60-60)
Male sex - n (%)	26 (81)	21 (81)	5 (83)	8 (75)
BMI - kg/m2 median (IQR)	28 (24-30)	29 (26-30)	25 (20-32)	-
Admission Glasgow Coma Score - points median (IQR)	3 (3-15)	3 (3-15)	15 (15-15)	
Days since hospital admission - median (IQR)	36 (14-47)	43 (22-52)	7 (5-11)	-
Days in ICU - median (IQR)	31 (19-43)	31 (19-43)	-	-
In-hospital mortality - n (%)	7 (21.9)	7 (26.9)	0 (0)	-
Glasgow Coma Score - points median (IQR)	3 (3-11.8)	3 (3-5.8)	15 (15-15)	
SOFA score - median (IQR)	7 (7-11)	7 (7-11)	-	-
LUMC severity score - median (IQR)	12 (9.8-14)	13 (12-14)	3 (1.5-3.8)	-
Comorbidities				
Chronic cardiac disease - n (%)	7 (21.9)	6 (23.1)	1 (16.7)	-
Hypertension - n (%)	8 (25)	7 (26.9)	1 (16.7)	-
Chronic pulmonary disease - n (%)	3 (9.4)	3 (11.5)	0 (0)	-
Asthma - n (%)	6 (18.8)	6 (23.1)	0 (0)	-
Chronic kidney disease - n (%)	1 (3.1)	1 (3.9)	0 (0)	-
Chronic neurological disorder - n (%)	3 (9.4)	2 (7.7)	1 (16.7)	-
Diabetes	13 (40.6)	9 (34.6)	4 (66.7)	-
Smoking history - n (%)	10 (31.3)	10 (38.5)	0 (0)	-
Thromboembolic event - n (%)	4 (12.5)	4 (15.4)	0 (0)	-
Coagulopathy - n (%)	1 (3.1)	1 (3.9)	0 (0)	-
Respiratory function				
Respiratory rate - median (IQR)	26.5 (24-30)	28.5 (25-32)	20.5 (17-23)	-
SpO2 - % median (IQR)	90 (89-92)	89.5 (88-90)	94.5 (94-96)	-
FiO2 - % median (IQR)	60 (41-100)	60 (41-100)	-	-
PaO2/FiO2 ratio - median (IQR)	17 (12.7-20.8)	17 (12.7-20.8)	-	-
Non-invasive ventilation - n (%)	8 (25)	7 (27)	1 (17)*	-
Invasive ventilation - n (%)	17 (53)	16 (62)	1 (17)*	-
Coagulation				
Platelets - 10^9/L median (IQR)	230 (200-302)	2245 (194-309)	253 (223-274)	-
aPTT - seconds median (IQR)	37.5 (33.6-39.9)	37.8 (34.4-40.3)	33.5 (31.5-35.5)	-
PT - seconds median (IQR)	16.1 (14.8-16.8)	15.6 (14.7-16.7)	16.65 (16.6-16.7)	-
INR - median (IQR)	1.2 (1.1-1.2)	1.2 (1.1-1.2)	1.2 (1.2-1.2)	-
D-dimer - mg/L median (IQR)	2.24 (1.03-3.96)	2.24 (1.03-3.96)	-	-
Inflammation markers				
CRP - mg/L median (IQR)	164.4 (58.5-245.1)	185.5 (55.1-255.5)	128.8 (76.6-170.9)	-
Urea - mmol/L median (IQR)	13.4 (5.9-16.8)	15.2 (11.5-17.1)	5 (4.5-5.2)	-

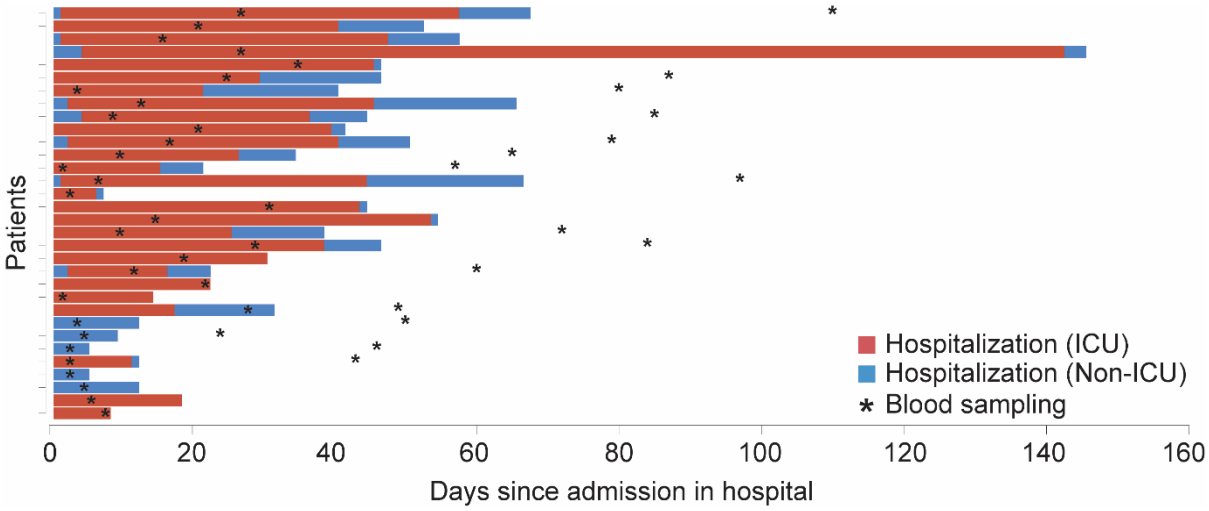
* This patient was first in ICU when treated with non-invasive or invasive ventilation, and stopped ventilation when in non-ICU unit. Blood sampling for this patient was during non-ICU hospitalization.

BMI, body mass index; IQR, interquartile range

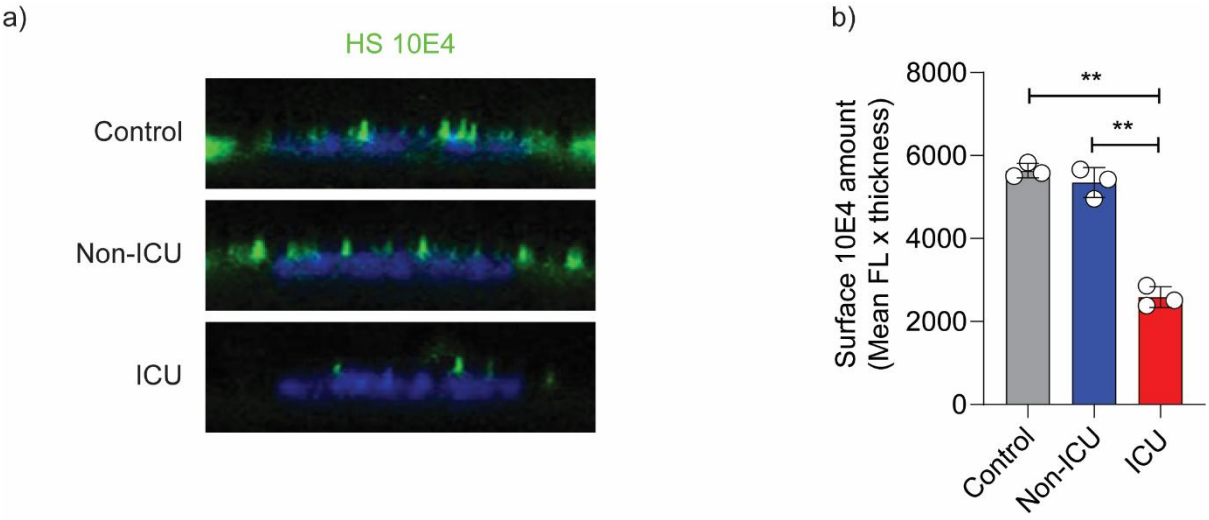
Supplementary table S2 Primer sequences

Gene	Forward sequence	Reverse sequence
GAPDH	CCTGCACCACCAACTGCTTA	GGCCATCCACAGTCTTCTGAG
HPSE	TCCTGCGTACCTGAGGTTTG	CCATTCCAACCGTAACTTCTCCT
SDC1	CCACCATGAGACCTCAACCC	GCCACTACAGCCGTATTCTCC
ICAM1	GTATGAACTGAGCAATGTGCAAG	GTTCCACCCGTTCTGGAGTC
ANGPT2	CTCGAATACGATGACTCGGTG	TCATTAGCCACTGAGTGTTGTTT
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
F3 (TF)	CCCAAACCCGTCAATCAAGTC	CCAAGTACGTCTGCTTCACAT

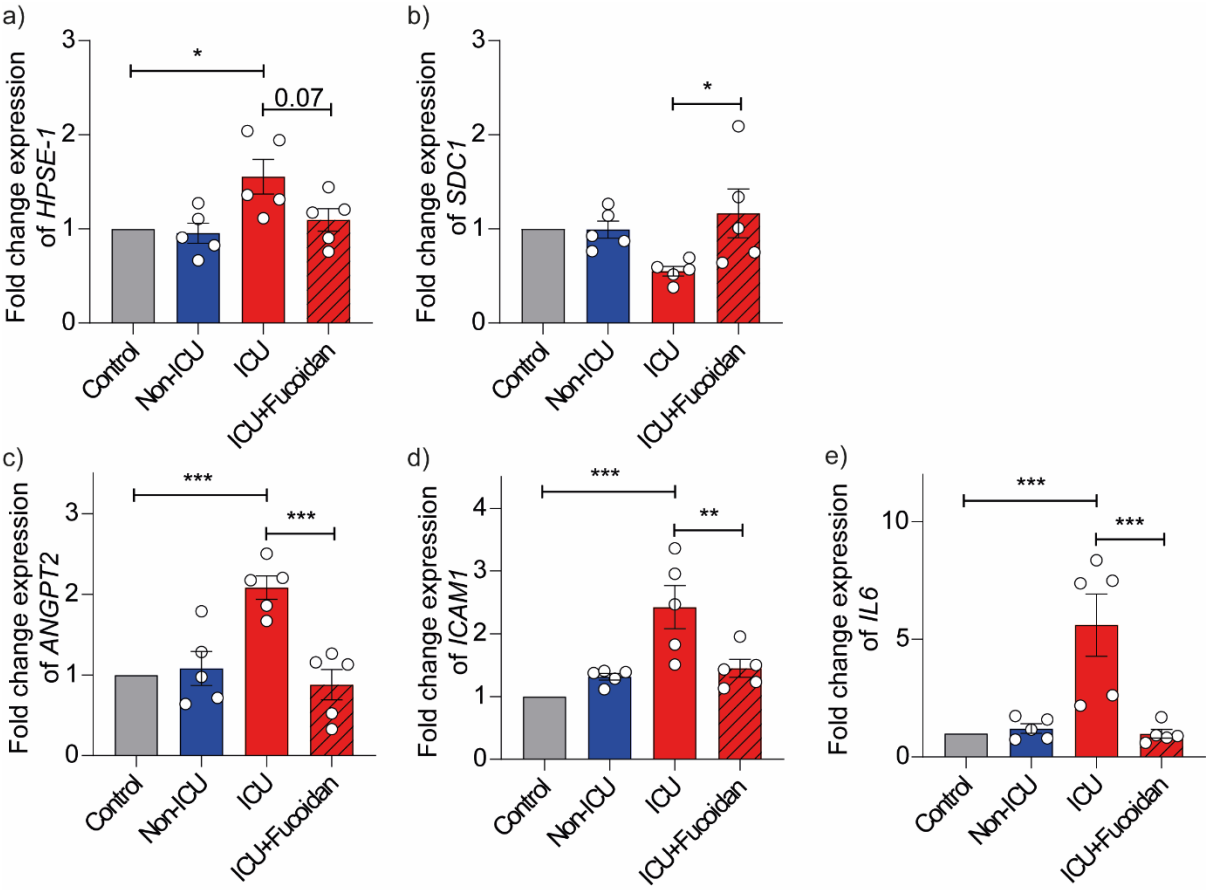
Supplementary figures



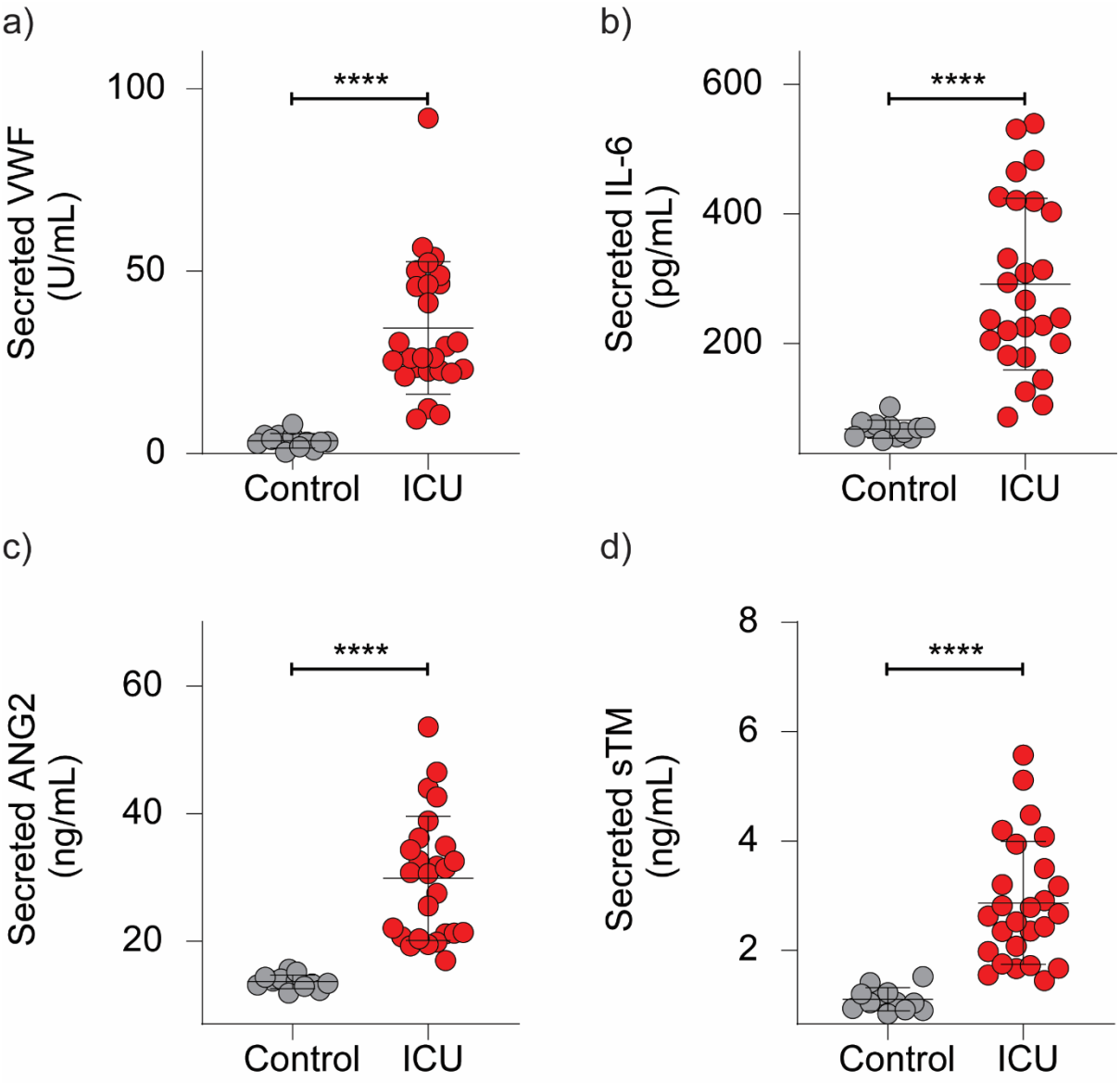
Supplementary figure S1 Patient's hospitalization timelines and blood sampling dates. For each included patient, hospitalization on non-ICU (blue bar), and on ICU (red bar) are indicated, aligned to the day of admission. * represent blood sampling date. For some of the patients, blood samples were collected during the whole hospitalization (ICU and non-ICU) and recovery state (discharge after 6 weeks).



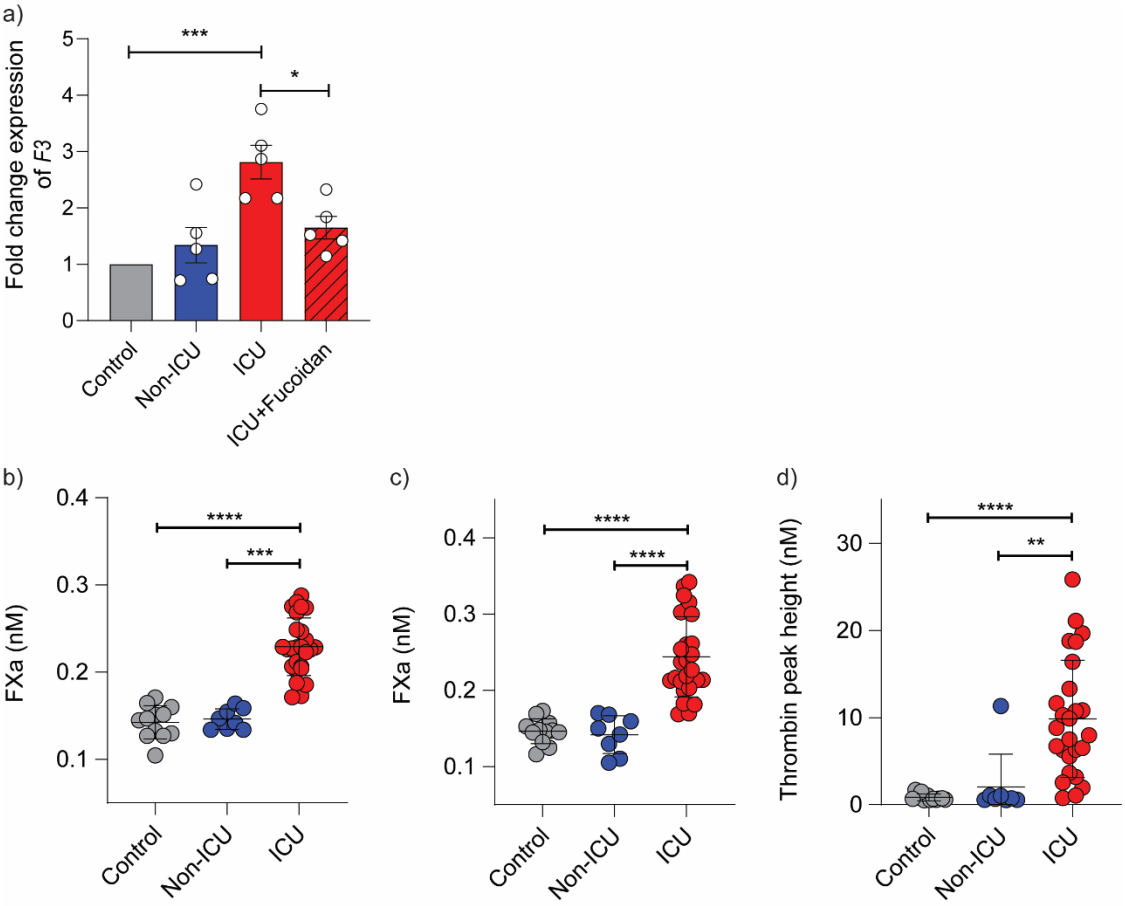
Supplementary figure S2 Loss of heparan sulfate in primary human pulmonary microvascular endothelial cells in presence of COVID-19 ICU serum. a) Representative confocal images of anti-heparan sulfate (HS, 10E4 clone) staining on the surface of primary human pulmonary microvascular endothelial cells (HPMECs) in the presence of 10% pooled- healthy control (n = 12), COVID-19 non-ICU (n = 8) and COVID-19 ICU (n = 26) serum for 24hrs. b) Quantification of HPMECs surface HS (10E4 clone) in the presence of 10% pooled- healthy control, COVID-19 non-ICU and COVID-19 ICU serum for 24hrs, presented as mean fluorescence times thickness of 3 independent experiments. Graphs represent the mean ± SD. One-way ANOVA followed by Tukey's multiple comparisons test were performed; **p<0.01



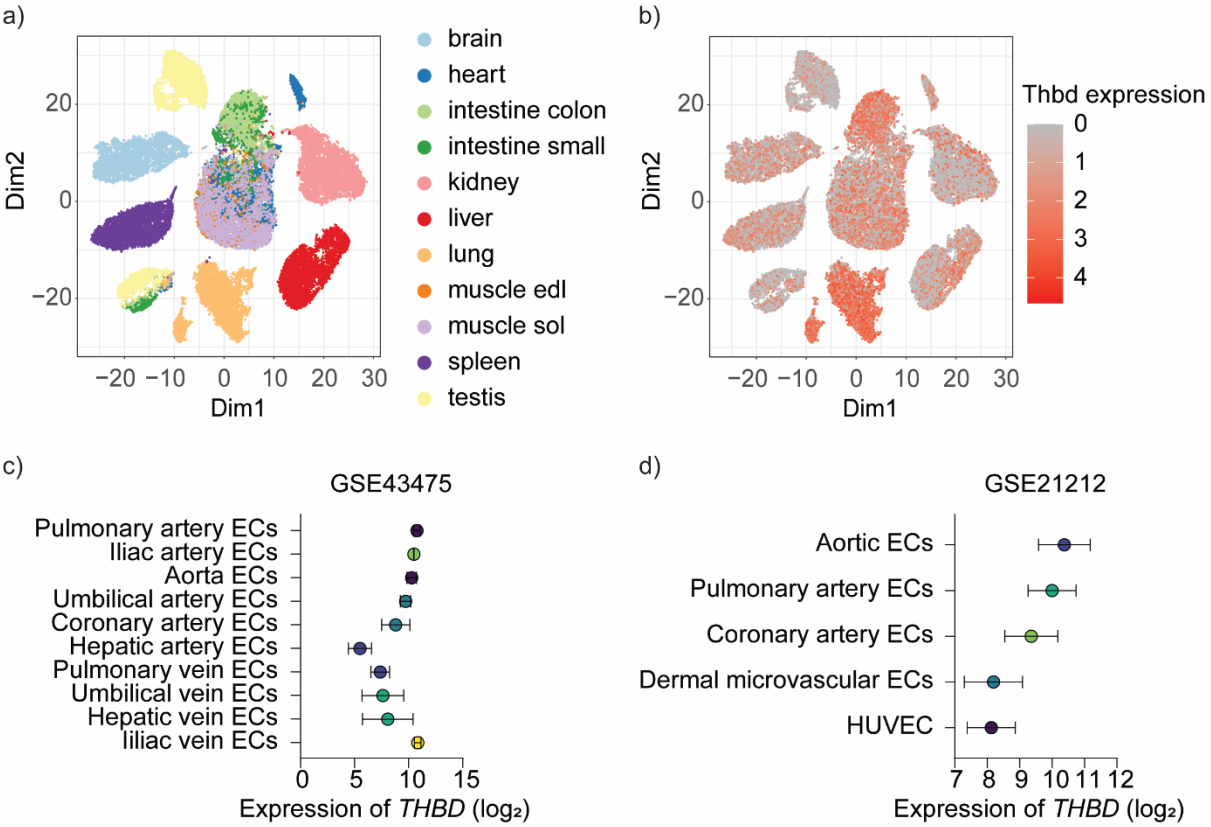
Supplementary figure S3 Fucoidan reduces mRNA expression of endothelial activation related markers. Gene expression data a) heparanase (*HPSE-1*), b) syndecan-1 (*SDC1*), c) *ICAM1*, d) angiopoietin 2 (*ANGPT2*) and e) *IL6* expression in response to 10% pooled- healthy control (n = 12), COVID-19 non-ICU (n = 8) and COVID-19 ICU (n = 26) serum with and without fucoidan (10 µg/mL) for 24hrs, presented as fold change expression normalized to healthy control of 5 independent experiments. Graphs represent the mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test were performed; *p<0.05, **p<0.01, ***p<0.001



Supplementary figure S4 Comparison of endothelial dysfunction and glycocalyx shedding related markers in cell culture supernatants. Levels of a) secreted von Willebrand factor (VWF), b) secreted IL6, c) secreted angiopoietin 2 (ANG2) and d) secreted soluble thrombomodulin (sTM) in cell culture supernatants of primary human pulmonary microvascular endothelial cells (HPMECS) in the presence of serum of healthy controls (grey, n = 12) and COVID-19 ICU patients (red, n = 26). Nonpaired two-tailed Student t test were performed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary figure S5 Serum mediators of COVID-19 patients on ICU promote activation of coagulation on endothelial cells. a) Gene expression of F3 (tissue factor) in response to 10% pooled healthy control (n = 12), COVID-19 non-ICU (n = 8) and COVID-19 ICU (n = 26) serum with and without fucoidan (10 µg/mL) for 24hrs, presented as fold change expression normalized to healthy control of 5 independent experiments. Factor X a (FXa) production (nM) in b) first hour and c) second hour on HPMECs surface in the presence of 10% individual healthy control (n=12), COVID-19 non-ICU (n=8) and COVID-19 ICU (n=26) serum for 24hrs . d) Thrombin generation peak height (nM) measured on HPMECs surface in the presence of 10% individual healthy control (n=12), COVID-19 non-ICU (n=8) and COVID-19 ICU (n=26) serum for 24hrs . One-way ANOVA followed by Tukey's multiple comparisons test were performed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplementary figure S6 Thrombomodulin expression levels in different vascular beds. a) tSNE plot revealing gene variation in 11 types of endothelial cells from mice based on EC atlas (https://endotheliomics.shinyapps.io/ec_atlas/). b) Thrombomodulin (*Thbd*) gene expression in 11 types of endothelial cells from mice. *THBD* expression from different human vascular beds based on databases c) GSE43475 and d) GSE21212.