SUPPLEMENT DATA

MIFlowCyt-EV of research study " Circulating platelet-derived extracellular vesicles are increased in severe COVID-19 disease "

1 Flow cytometry

1.1 Experimental design

The aim of flow cytometry (A50-Micro, Apogee Flow Systems, Hemel Hempstead, UK) was to compare the concentrations of circulating extracellular vesicles (EVs) of plasma from COVID-19 patients and healthy controls. All samples were measured using an autosampler, which facilitates subsequent measurements of samples in a 96-well plate. The entire study involved five 96-well plates that were measured within 1 month. Each well plate contained a buffer-only control. Light scatter and flow rate calibrations was performed each experiment day. Fluorescence calibration was done prior to the experiments for all the fluorescence detectors once.

1.2 EV staining

Circulating plasma EVs were stained with antibodies and lactadherin. Table S1 shows an overview of the used reagents and antibody concentrations used for staining. Each sample was double labelled with anti-CD31-PE- (phycoerythrin) and anti-CD45-FITC (fluorescein isothiocyanate), anti-CD61-PE and anti-CD235a-FITC, anti-mouse IgG1-PE and anti-mouse IgG1-FITC κ isotype control, and single labelled with lactadherin-FITC. To stain, 20 µL of pre-diluted plasma was incubated with 2-2.5 µL of antibodies or isotype controls, or 3.15 µl of lactadherin-FITC, and kept in the dark for 2 h at room temperature. The staining reaction was stopped by adding 200 µL of HEPES buffer.

1.3 Buffer-only control

Each 96-wellplate contained at least 1 well with filtered (0.1µm Millex.VV filtered) HEPES buffer, which was measured with the same flow cytometer and acquisition settings as all other samples. The mean count rate was below 50 events per second, which is substantially lower than the target count rate (1000-4000 events per second) for PEV samples.

1.4 Buffer with reagents control

Buffer with reagent control for each reagent was measured once prior to experiment with the same flow cytometer and acquisition settings as all other samples. For all reagents the mean count rate was below 50 events per second, which is similar to the buffer-only control.

1.5 Unstained controls

We diluted each sample 10-fold in HEPES buffer (140 mM NaCl containing 10 mM Hepes pH 7.4) and measured the total concentration of particles for 60 seconds with and without staining. For all experiments, HEPES buffer was filtered (0.1µm filter unit, Millex-VV, Merch Millipore).

1.6 Trigger channel and threshold

Based on the buffer-only control (48 events s-1), the trigger was set at 12 arbitrary units SSC, which is equivalent to a side scattering cross section of 25 nm2 (Rosetta Calibration, v1.11, Exometry, Amsterdam, The Netherlands).

1.7 Flow rate quantification and light scatter calibration

Apogee Bead Mix (Apogee flow systems, UK) was used to quantify the flow rate, and Rosetta Calibration (Exometry, The Netherlands) to relate light scatter measured by FSC or SSC to the scattering cross section and diameter of EVs. Circulating EVs were modelled as core-shell particles with a core refractive index of 1.38, a shell refractive index of 1.48, and a shell thickness of 6 nm. For each measurement, we added the FSC

and SSC scattering cross sections and EV diameters to the flow cytometry datafiles by Rosetta calibration software. OneComp ebeads (Thermo Fisher Scientific) was used to compensate the spectral overlap of PE and FITC according to manufacturer's instructions using anti-human CD61 PE (clone VI-PL2, BD Bioscience) and anti-human CD41 FITC (clone HIP8, BD Bioscience). Each single-stained bead sample was run with Apogee with the same fluorescent settings as the samples to perform compensation setup that was then used for the PE-FITC double-labelled samples.

1.8 EV number concentration

The concentrations of circulating mEVs, lEVs, and CR reported in the manuscript describe the number of particles (1) that exceeded the SSC threshold, corresponding to a side scattering cross section of 25 nm2, (2) with a diameter >200 nm, (3) fulfilling the condition of equation S2 and, (4) are positive at the corresponding fluorescence detector(s), per mL of plasma. The representative gating strategy is presented in figure S1.

1.9 Data sharing

All data including summary of all flow cytometry scatter plots and gates applied, as well as raw data and data with standard units will become available upon request (mari.palviainen@helsinki.fi).

2 References

[1] van der Pol E, van Gemert MJC, Sturk A, Nieuwland R, van Leeuwen TG. Single vs. swarm detection of microparticles and exosomes by flow cytometry. J Thromb Haemost 2012; 10: 919–30.

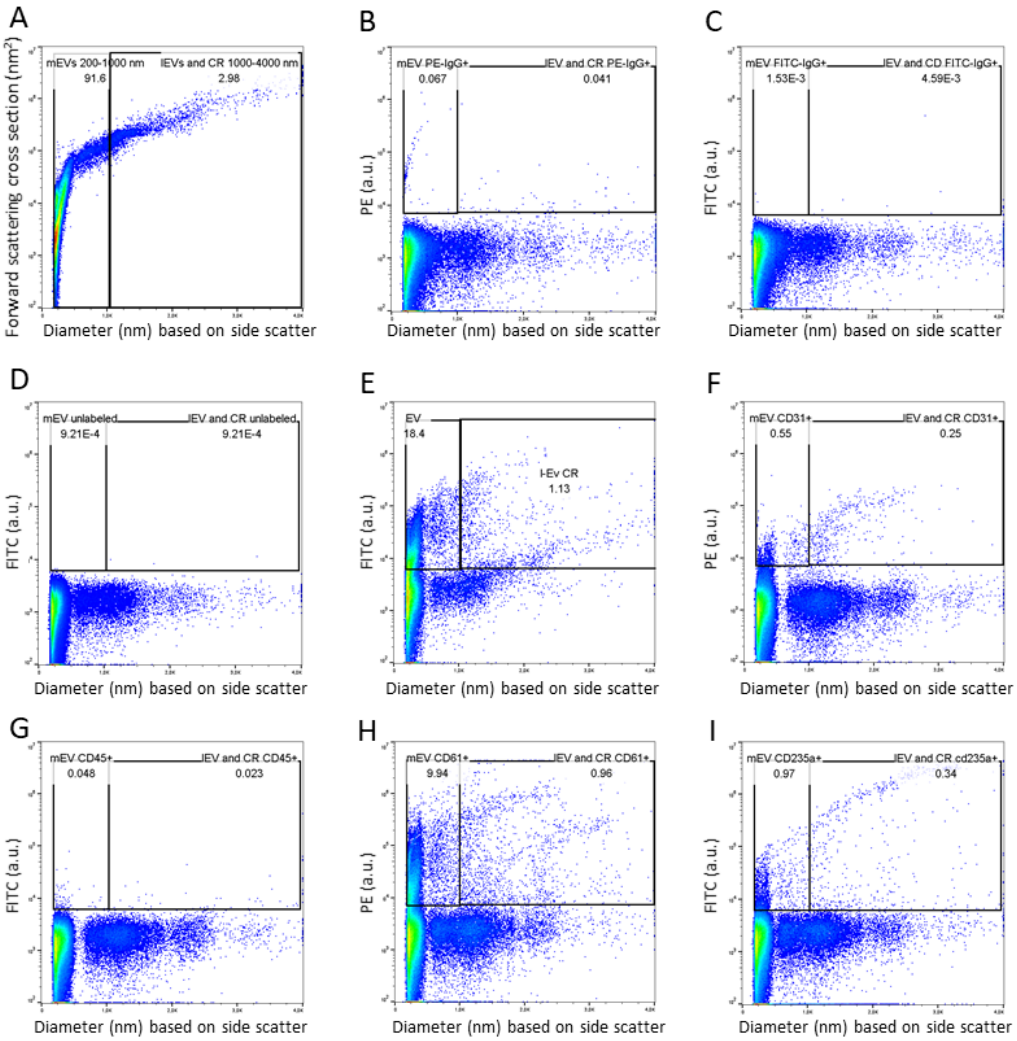
[2] van der Pol E, de Rond L, Coumans FAW, Gool EL, Böing AN, Sturk A, Nieuwland R, van Leeuwen TG. Absolute sizing and label-free identification of extracellular vesicles by flow cytometry. Nanomedicine Nanotechnology, Biol Med 2018; 14: 801–810

Table S1: Overview of staining reagents. The antibody concentration during measurements was 11.1-fold lower than the antibody concentration during staining.

Antibody	Clone	cat nro/supplier	Used dilution
PE Mouse Anti-Human CD31	WM59	555446/BD Pharmigen	2.5 µl to 20 µl sample
FITC Anti-human CD45	HI30	555482/BD Pharmingen	2.5 µl to 20 µl sample
PE Mouse Anti-Human CD61	VI-PL2	555754/BD Pharmimgen	2.5 µl to 20 µl sample
FITC CD235a, Glycophorin A	JC159	F0870/Agilent	2 µl to 20 µl sample
FITC Mouse IgG1, κ Isotype Control	NA	555909/BD Pharmimgen	2.5 µl to 20 µl sample
PE Mouse IgG1, κ Isotype Control	NA	555749/BD Pharmimgen	2.5 µl to 20 µl sample
FITC lactadherin (bovine)	NA	Haematological Technologies	3.15 µl to 20 µl reaction

APC: allophycocyanin; CD: cluster of differentiation; FITC: fluorescein isothiocyanate; IgG: immunoglobulin G; PE: phycoerythrin.

Figure S1. The representative gating strategy of mEVs, lEVs, and CR.



Gating strategy for medium sized EVs (mEV), large EVs (lEV) ja cell remnants (RC) flow cytometry analysis. Representative dot plots are shown for unstained (D), mouse IgG1-PE (B), mouse-IgG1-FITC (C), mouse anti-human CD31-PE (F), anti-human CD45-FITC (G), anti-human CD61-PE (H), anti-human CD235a (I), lactadherin-FITC (E) stained mEVs, lEVs and CR from plasma. mEVs, lEVs and CR were detected based in their light scatter calibrated with Rosetta calibration system (Exometry, The Netherlands) to set a mEV diameter gate of 200-1000 nm (A), and CR diameter gate of 1000-4000 nm. Next fluorescence gate was set using unstained PEVs (D). Positive events (+) were defined as events with fluorescent signal exceeding the threshold. Isotype control (B&C) was used to differentiate between specific and nonspecific binding of antibodies.

Table S2: Summary of the laboratory tests results of the 50 patients with COVID-19 infection, divided by D-dimer cut-off 1,5 mg/L used for grouping to severe and non-severe COVID-19 infection.

	D-dimer	median	range	interquartile range	reference range	NA's
PT (%)	**≤1,5 mg/L**	102	23-133	94-112	70-130	1
	>1,5 mg/L	90	50-115	83-100		0
APTT (s)	**≤1,5 mg/L**	35	30-138	33-39	28-37	4
	>1,5 mg/L	34	27-76	32-40		0
Fibrinogen (g/L)	**≤1,5 mg/L**	5.6	0.4-9.5	4.3-7.2	2.0-4.0	0
	>1,5 mg/L	6.5	2.3-11.5	4.8-7.7		0
FVIII (IU/mL)	**≤1,5 mg/L**	128	35-266	84-177	60-160	2
	>1,5 mg/L	261	119-489	213-270		0
Thrombin time (s)	**≤1,5 mg/L**	20	16-35	19-23	17-25	3
	>1,5 mg/L	24	22-37	23-27		4
VWF:Act (IU/dL)	**≤1,5 mg/L**	212	10-432	79-275	50-190	0
	>1,5 mg/L	354	113-563	264-480		0
VWF:Ag (IU/dL)	**≤1,5 mg/L**	332	135-874	229-417	50-190	0
	>1,5 mg/L	496	201-786	427-634		0
VWF:CB (IU/dL)	**≤1,5 mg/L**	143	4-328	21-199	55-180	8
	>1,5 mg/L	222	16-530	194-330		0
Antithrombin (IU/dL)	**≤1,5 mg/L**	90	7-137	65-107	85-125	2
	>1,5 mg/L	84	33-106	74-98		1
D-dimer (mg/L)	**≤1,5 mg/L**	0.5	0.2-1.3	0.3-0.7	<0.5	0

	>1,5 mg/L	3.6	1.6-11.3	2-5.1		0
Plt (x10^9/L)	**≤1,5 mg/L**	269	144-694	213-399	150-360	0
	>1,5 mg/L	416	93-689	332-515		1
Anti-Xa (IU/mL)	**≤1,5 mg/L**	0.08	0-0.59	0.02-0.13	-	5
	>1,5 mg/L	0.23	0-0.42	0.15-0.3		1
Hgb (g/L)	**≤1,5 mg/L**	124	95-147	115-131	[1]	1
	>1,5 mg/L	98	73-124	89-102		1
Leuk (x10^9/L)	**≤1,5 mg/L**	5.5	2-20.6	4.7-8.2	3.4-8.2	0
	>1,5 mg/L	9.7	4.2-16.1	7.1-11.9		1
CRP (mg/L)	**≤1,5 mg/L**	48	4-230	13-107	<4	1
	>1,5 mg/L	84	20-398	37-122		1
ADAMTS13 (%)	**≤1,5 mg/L**	82	19-140	55-99	60-130	4
	>1,5 mg/L	54	29-107	41-63		0
CAT Peak (nM)	**≤1,5 mg/L**	119	4-325	43-149	142-178[2]	5
	>1,5 mg/L	20	2-253	8-56		2
CAT ETP (nM*min)	**≤1,5 mg/L**	1116	265-2030	631-1515	1140-1391[2]	8
	>1,5 mg/L	615	109-2174	352-818		7
CAT LT (min)	**≤1,5 mg/L**	5.3	3-18.5	3.9-6	3.00-3.67[2]	5
	>1,5 mg/L	7.2	2.7-41.5	5.5-11.8		2

1 Hgb ref interval 134-167 g/L for men, 117-155 g/L for women. 2 range of CAT TG from normal plasma

Figure S2: While von Willebrand factor activity correlated with von Willebrand factor antigen (R^2=0.52) and von Willebrand factor collagen binding assay (R^2=0.38), the correlation with ADAMTS13 activity (R^2=0.01) with von Willebrand factor activity and antigen (R^2=0.11) were poor

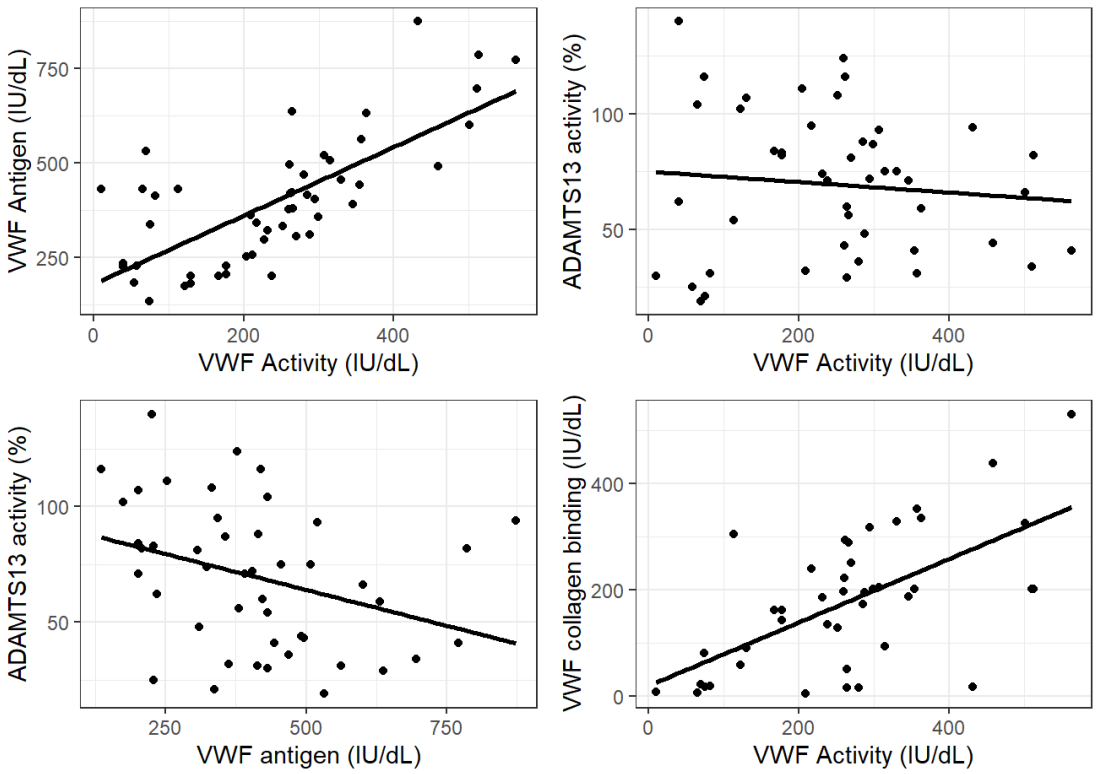


Figure S3. Coagulation tests, C reactive protein levels and platelet counts at D-dimer levels of > 1.5 mg/l compared with D-dimer ≤ 1.5 mg/L. Von Willebrand factor (VWF) activity and antigen levels were significantly higher at high D-dimer levels (p<0.001), as well as ADAMTS13 lower (p=0.02). Platelet count was significantly higher (p=0.04). C reactive protein (p=0.13) and fibrinogen (p=0.44) levels did not differ. Box plots, show median, 25th and 75th percentiles and range. Local reference intervals are shown (dashed lines)

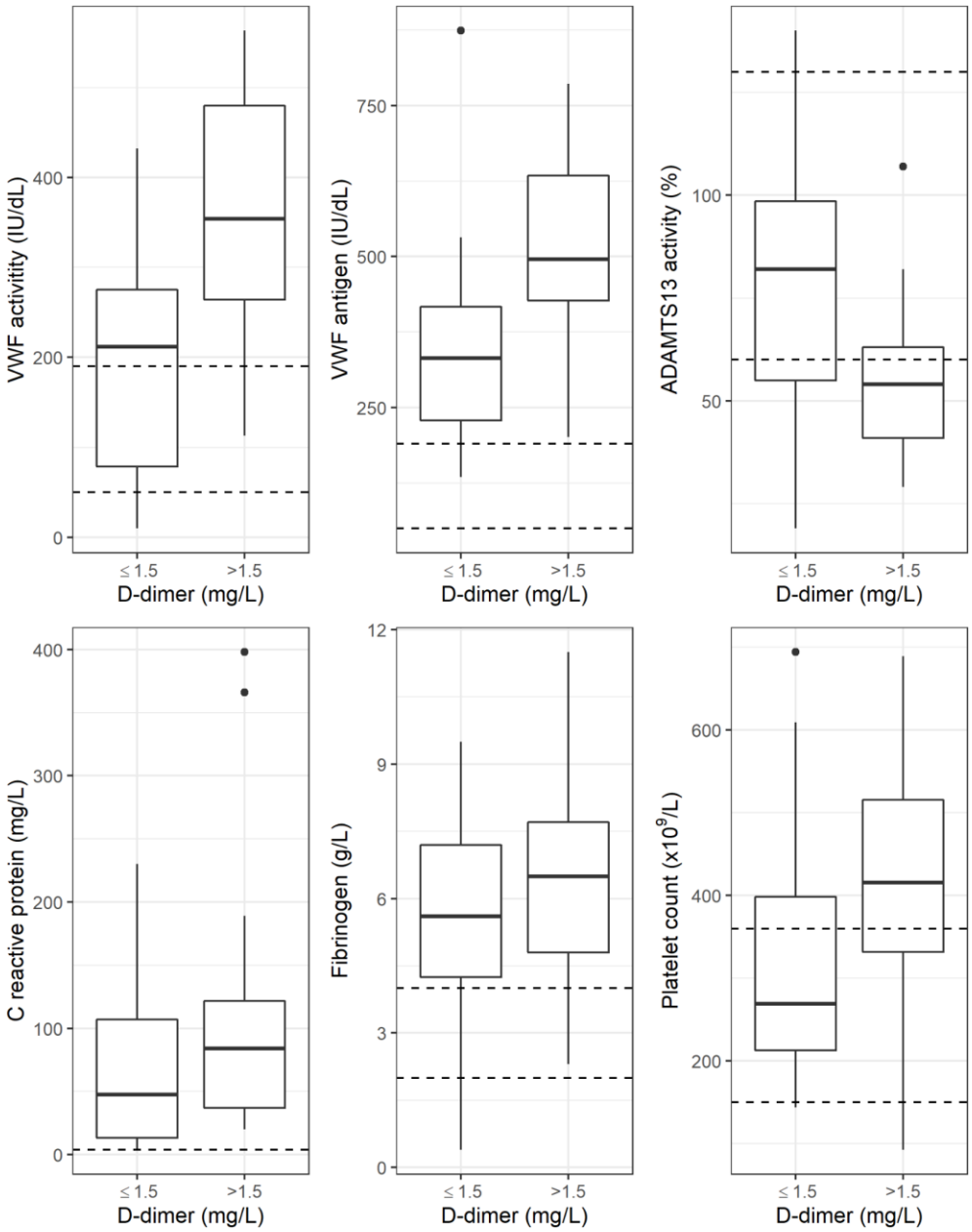


Figure S4

Thrombin generation in plasma of COVID-patients grouped by D-dimer levels. Lag time and Time to Peak were increased and ETP and Peak medians below the normal range in these COVID-patients (pooled plasma median, solid line; range, dashed lines). Only Peak and Time to Peak were different between D-dimer groups, with D-dimer over 1.5 mg/l, group having longer Time to Peak (p=0.04) and lower Peak (p=0.007). One sample with Lag time over 40 min, is not shown in Lag Time -figures. Of the patients, three had such high amount of thrombin generated that the thrombin substrate was consumed during the analysis, and thrombin peak was not obtained. Pairwise comparisons were determined using Mann Whitey U test, with Bonferroni correction.