Supplementary materials

NETosis induction reflects COVID-19 severity and Long COVID: insights from a two-center patient cohort study in Israel

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1 **Supplemental methods**
2 **ABO reverse grouping.** To determine blood type, plasma samples were tested
3 against red cell reagents (Referencells®-4, IMMUCOR) and inspected visually
4 for agglutination occurrence.
5
6 **Platelet activation marker and coagulation factor assays.** PF4 and soluble
7 P-selectin (sP-selectin) plasma levels were measured using the Quantikine®
8 ELISA Human CXCL4/PF4 and Quantikine® ELISA Human P-Selectin/CD62P
9 Immunoassays (DPF40 and DPSE00, respectively, R&D Systems, Inc.). VWF
10 and FVIII (total FVIII antigen) plasma levels were measured using ELISA kits
11 per manufacturer protocol (ab108918 and ab272771, respectively, Abcam).
12 Due to insufficient sample volumes, FVIII plasma levels could not be
13 determined for 1 non-COVID subject,
14
15 **MPO antigen measurements.** MPO plasma levels were measured using the
16 LEGEND MAX™ Human Myeloperoxidase ELISA Kit (440007, BioLegend
17 Inc.). Due to insufficient volume, levels could not be determined for 6 subjects
18 (3 COVID-19 subjects, 1 convalescent and 2 non-COVID).
19
20 **H3Cit-DNA ELISA.** H3Cit-DNA complex plasma levels were evaluated using a
21 sandwich ELISA-based assay as previously described.[26] Immulon 4 HBX-well
22 plates (3855, Thermo Scientific™) were coated with recombinant anti-histone
23 H3 (citrulline R8) antibody (ab232939, Abcam), washed, and the plate blocked
24 with 1% BSA in PBS. After washing, plasma and standard samples
25 (H3Cit2,8,17 dNuc nucleosomes (16-1362, EpiCypher®) were added to the
26 plate together with peroxidase-conjugated anti-DNA antibody (from Cell Death
27 Detection ELISA kit, 11544675001, Roche Diagnostics). The plate was
28 incubated for 2 hours at room temperature with agitation at 80 rpm, washed
29 and TMB One Component Microwell Substrate (0410-01L, Southern biotech™)
30 used for ELISA development, with 1M HCl used to stop the reaction.
31 Absorbance measurements at 450 nm with 620 nm reference wavelength were
32 used to plot a calibration curve and calculate H3Cit-DNA complex levels. All
33 samples from COVID-19 convalescent subjects were analyzed for H3Cit-DNA
34 complex levels, except for 1 sample due to insufficient sample volume.

35

36 **Cell-free DNA (cfDNA) quantification.** Plasma samples were diluted in RPMI
37 medium (01-100-1A, Biological Industries) supplemented with 0.5% 70°C heat-
38 inactivated fetal bovine serum (04-127-1A, Biological Industries), 10 mM
39 HEPES buffer (03-025-1C, Biological Industries) and 1 IU/ml of heparin
40 (289973.01-IL, Teva Medical Marketing). Lambda DNA standard (from the
41 Quant-iT™ Picogreen™ dsDNA Assay Kit, Invitrogen, P7589) was diluted
42 similarly and used to calculate a standard curve. The diluted samples were
43 mixed with 2 µM SYTOX Green dye (S7020, Invitrogen) in a 1:1 ratio. Then,
44 fluorescence levels (excitation 488 nm, emission 530 nm) were measured and
45 used to calculate values from the standard curve.

Table S1: Descriptive data of all study subjects and plasma samples.

n	Shaare Zedek Medical Center, n	Rambam Health Care Campus, n	Total, n
Study subjects			
Total	152	94	246
Non-COVID	10	44	54
Mild/moderate COVID-19	37	15	52
Severe-critical COVID-19	39	35	74
Convalescent[a]	66	-	66

[a]16 of the convalescent patients also have a plasma sample that was taken during acute COVID-19.

Table S2: Adjusted P values from Dunn's multiple comparisons tests of clinical and demographic characteristic of study cohorts.

Variable	Non-COVID-Mild/ moderate	Non-COVID-Severe/ critical	Non-COVID-Convalescents	Mild/ moderate-Severe/ critical	Mild/ moderate-Convalescents	Severe/ critical-Convalescents
Age	>0.9999	**<0.0001**	0.8661	**<0.0001**	>0.9999	**<0.0001**
BMI	>0.9999	**0.0172**	0.9190	0.1614	>0.9999	0.8233

Table S3: P values of multiple comparisons tests for platelet activation markers and coagulation factors, MPO-DNA and NETosis induction levels of study cohorts.

Variable	Non-COVID-Mild/ moderate*	Non-COVID-Severe/ critical*	Non-COVID-Convalescents**	Mild/ moderate-Severe/ critical*	Mild/ moderate-Convalescents*	Severe/ critical-Convalescents*
VWF	**0.0251**	**0.0023**	0.9591	>0.9999	**0.0255**	**0.0019**
FVIII	**0.0270**	**<0.0001**	**0.0012**	**<0.0001**	>0.9999	**<0.0001**
PF4	**0.0023**	**0.0001**	0.4934	>0.9999	**0.0001**	**<0.0001**
P-selectin	>0.9999	**<0.0001**	0.0862	**0.0041**	0.0775	**<0.0001**
MPO	**<0.0001**	**<0.0001**	0.1869	0.1211	**<0.0001**	**<0.0001**
cfDNA	**<0.0001**	**<0.0001**	>0.9999	0.0645	**<0.0001**	**<0.0001**
MPO-DNA complexes	0.7886	**0.0389**	0.2198	>0.9999	**0.0450**	**0.0001**
NETosis induction level	0.1621	**<0.0001**	0.4802	**0.0035**	0.4546	**<0.0001**
MPO-DNA complexes UDL occurrence* **	0.1178	0.8145	**0.0437**	**0.0497**	**0.0003**	0.0628

*** Adjusted P values according to unpaired Kruskal-Wallis test and Dunn's multiple comparisons test for multiple comparisons.**
**** P values according to two-tailed Mann–Whitney tests**
***** P values according to Fisher's exact tests. Same values for analysis with first sample of sequential samples.**

Table S4: Self-reported symptom list from Long COVID patients

Symptoms	Number of subjects experiencing the symptom
Fatigue	25
Dyspnea	18
Chest pressure/pain	8
Cough	7
Lung damage (chronic or acute)	8
Lost/changed sense of smell/taste	5
Hair loss	4
Difficulty concentrating	4
Muscle pain	4
Hypoxemia	3
Fast pulse	3
Nausea	2
Hypertension	2
Joint pain	2
Headache	1
Facialis	1
Sleeping difficulty	1
Shivering	1
Hearing damage	1
Pain during deep breathing	1
Burn in throat	1
Neck pain	1
Sleep apnea	1
Heart damage	1

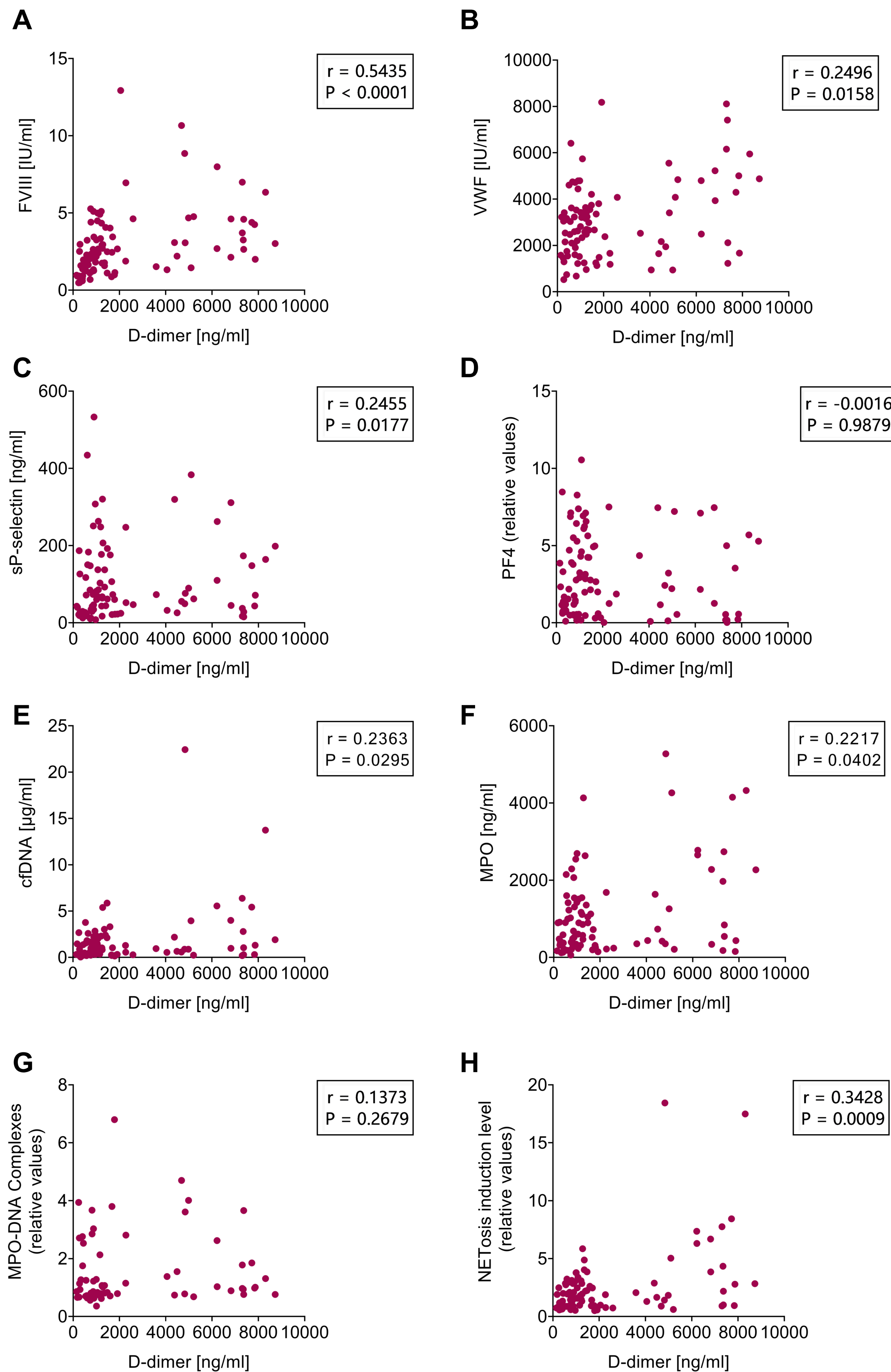

Figure S1: Correlations between D-dimer levels and platelet activation, coagulation factors and NET-related measurements of COVID-19 patient cohorts. (A) FVIII (n=93), **(B)** VWF (n=93), **(C)** P-selectin (n=93), **(D)** PF4 (n=93), **(E)** cfDNA (n=85), **(F)** MPO (n=86), **(G)** MPO-DNA complex levels (n=67) and **(H)** NETosis induction (n=90). Statistical analysis was performed using the two-tailed Spearman correlation test.

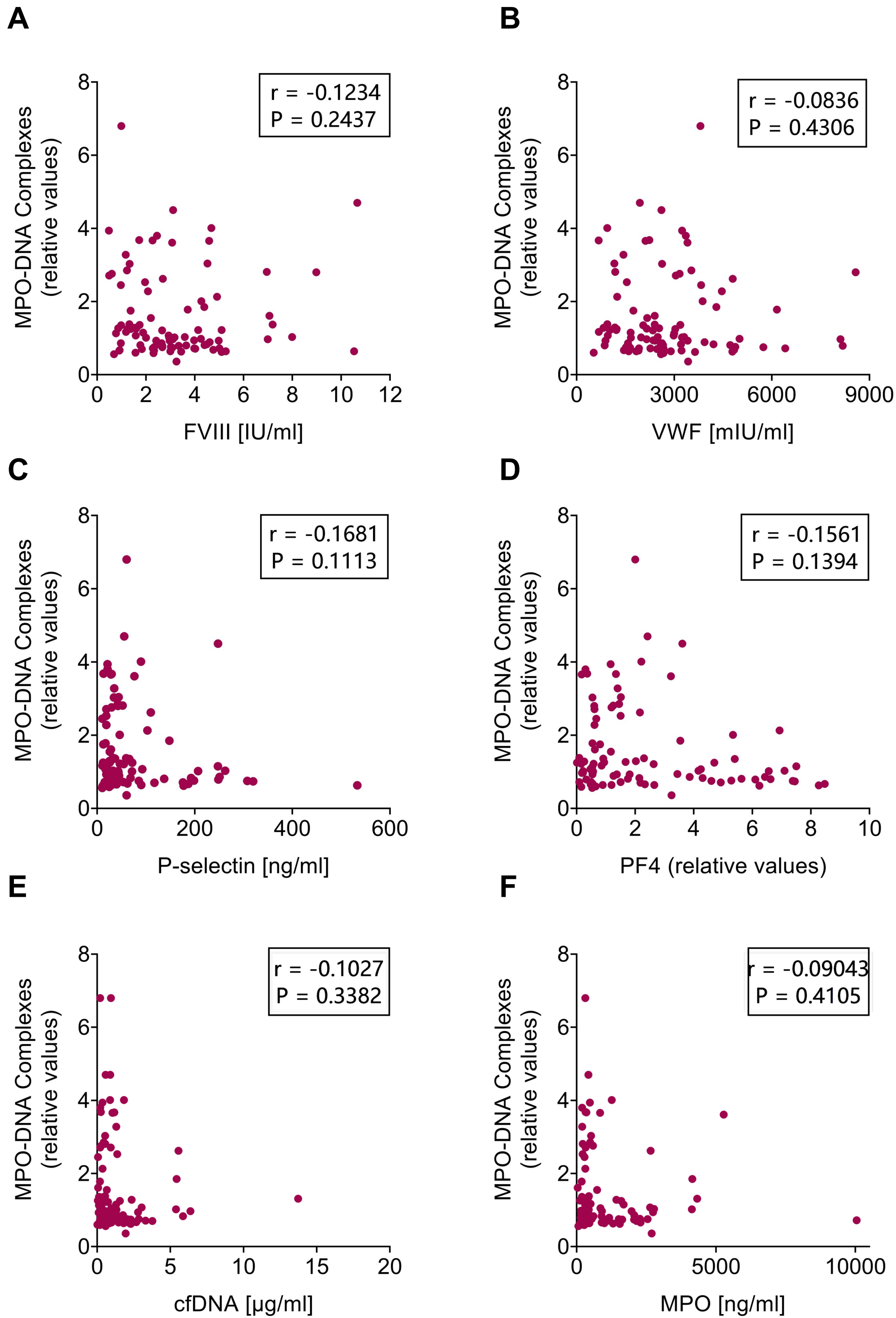

Figure S2: Correlations between MPO-DNA complex levels and platelet activation and coagulation factors of COVID-19 patient cohorts. (A) FVIII (n=91), **(B)** VWF (n= 91), **(C)** P-selectin (n= 91), **(D)** PF4 (n= 91), **(E**) MPO (n=85) and **(F**) cfDNA (n=89). For subjects with sequential samples, the maximal value is presented. Statistical analysis was performed using the two-tailed Spearman correlation test.

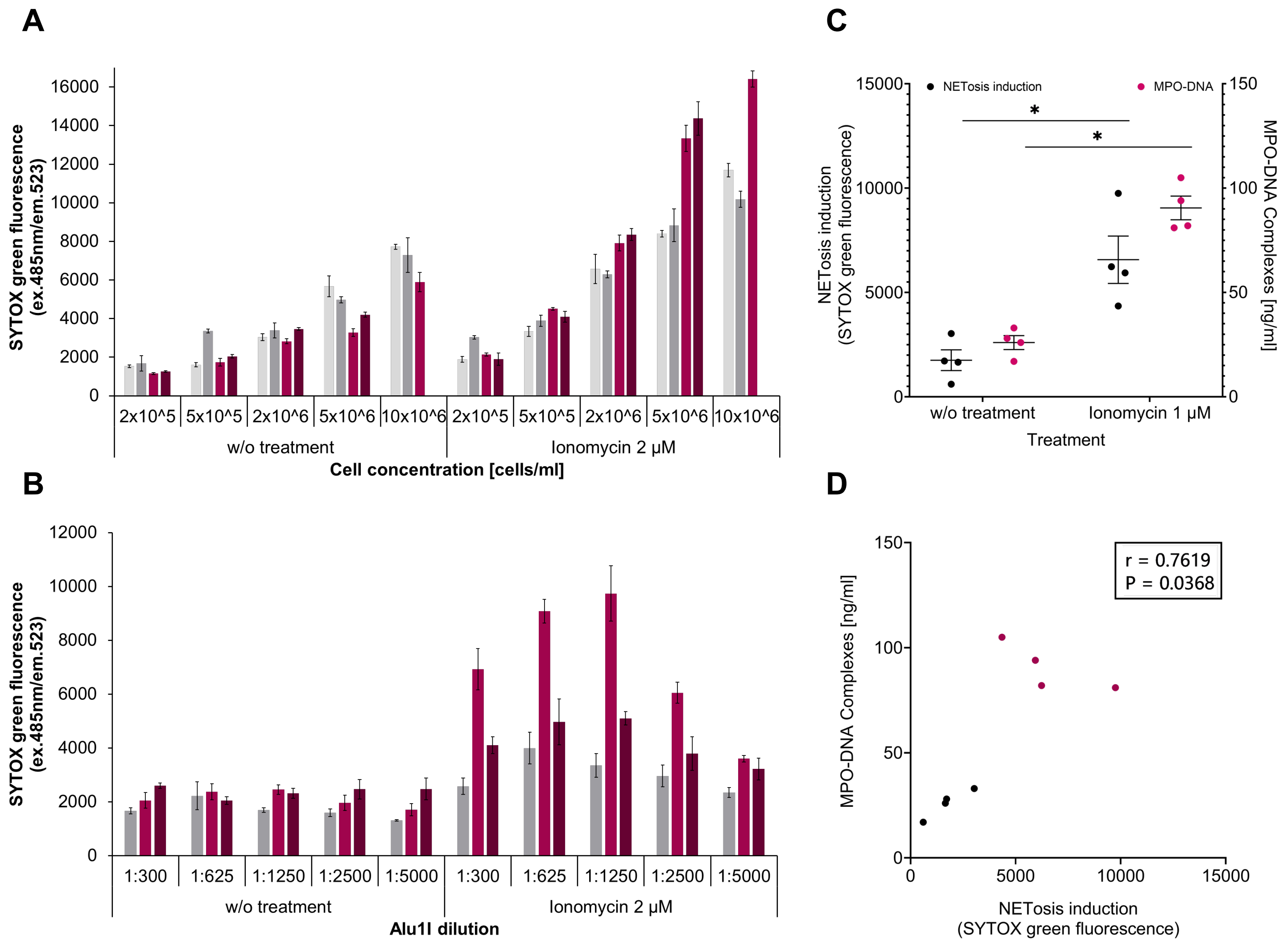

Figure S3: Optimization of NETosis induction assay conditions. NETosis induction was evaluated by incubating healthy neutrophils with and without 2 μM ionomycin treatments for 1 hr. **(A)** Different cell numbers seeded per well were evaluated in 4 experimental repetitions represented by different colors. Concentration of $2x10^6$ cells/ml resulted in the highest sensitivity, when comparing induced to untreated cells, and was used in following assay. **(B)** Different AluI dilutions were evaluated in 3 experimental repetitions represented by different colors. **(C)&(D)** Parallel MPO-DNA level and NETosis induction evaluation (using SYTOX green fluorescence readouts on digested samples) of the same samples. Dilution of 1:1250 resulted in the highest sensitivity, when comparing induced to untreated cells, and was used in following assay. **(C)** Significant induction was demonstrated by both quantification methods according to two-tailed Mann–Whitney tests with n=4 per group: MPO-DNA P = 0.0286 and NETosis induction P = 0.0286. **(D)** A significant positive correlation was found between MPO-DNA and NETosis induction in the assay samples. Statistical analysis was performed using by calculating the two-tailed Spearman correlation coefficient.

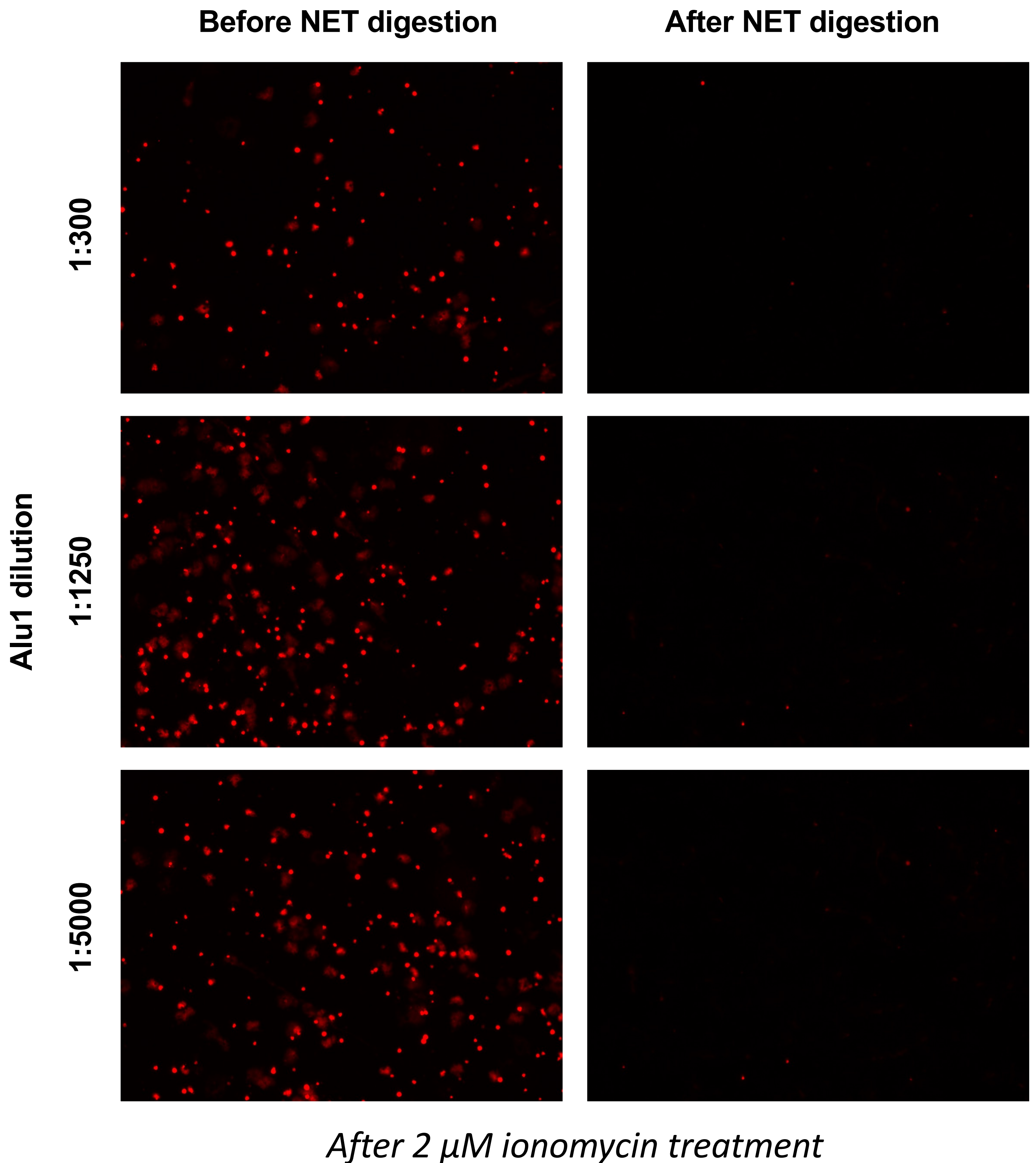

Figure S4: Confirmation of NET digestion using fluorescence microscopy imaging. NETosis induction in neutrophils ($2x10^5$ cells/well) was visualized with and without 2 µM ionomycin treatments in the presence of 0.2 µM of SYTOX orange. After a 1-hr incubation, wells were imaged, and then treated with different concentration of AluI to digest NETs into fragments that are released into supernatants. After a 20-minute incubation, wells contents were removed, and wells were visualized to check for residual SYTOX signal. Analysis was performed using EVOS M5000 imaging system (Thermo Fisher Scientific), 20X magnification with RFP filter.

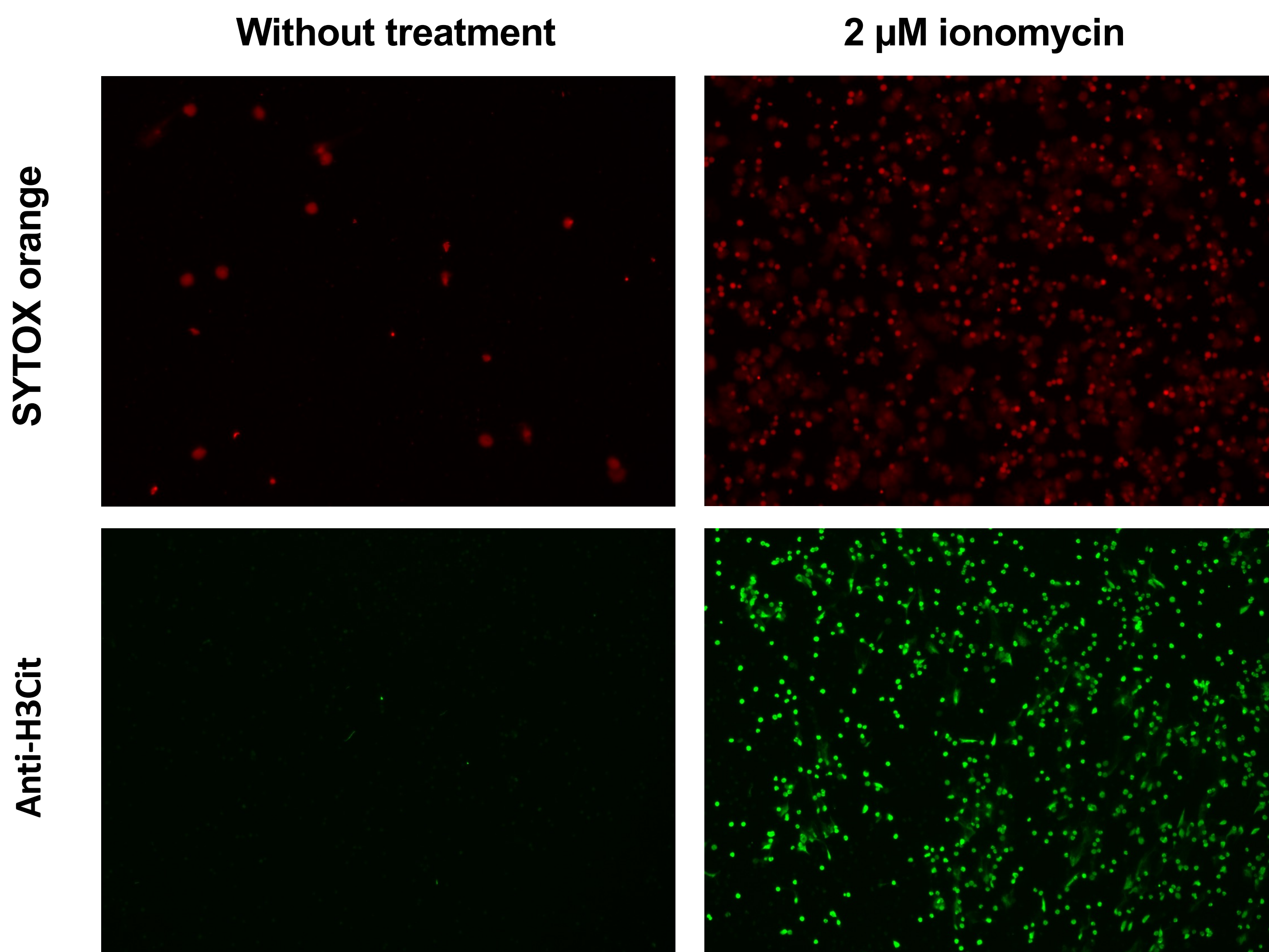

Figure S5: Validation of SYTOX signal with NET-specific immunostaining for citrullinated histones. NETosis induction was confirmed by SYTOX orange staining of neutrophils ($1.9x10^5$ cells/well) 1 hr after incubation with agonists (red signal). In addition, cells were fixed using 2% PFA and immunofluorescent staining was performed with anti-Histone H3 (citrulline R2 + R8 + R17) (H3Cit) as primary antibody and donkey anti-rabbit IgG (H+L) AF488 (green signal) as secondary antibody. Analysis was performed using the EVOS M5000 imaging system (ThermoFisher Scientific), 20X magnification with RFP and GFP filters.

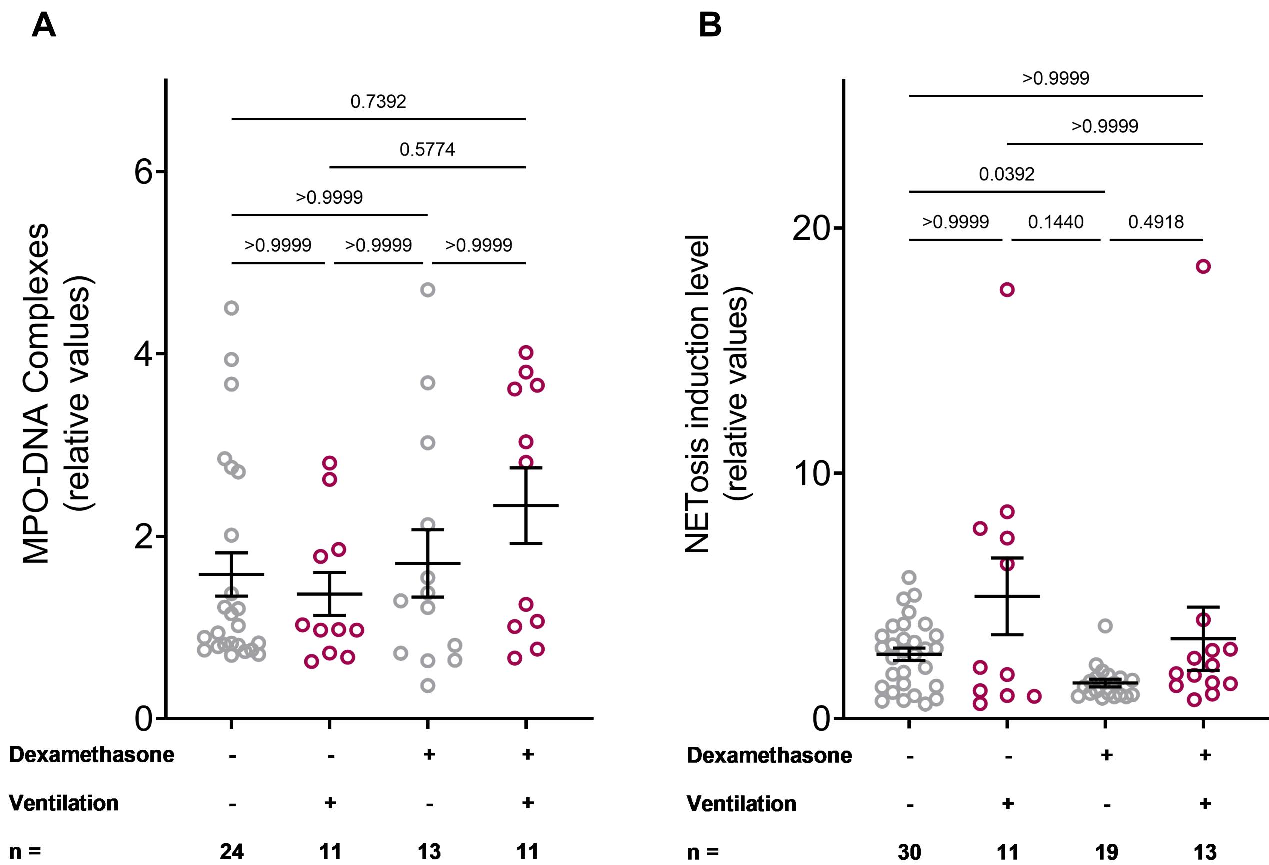

Figure S6: Subgroup analysis of severe/critical COVID-19 subjects with or without dexamethasone treatment or ventilation. (A) MPO-DNA complex levels **(B)** NETosis induction levels. MPO-DNA levels for 12 severe/critical COVID-19 subjects were under detection limits and are not presented in the graph. Due to limited sample volume NETosis induction levels could not be generated for one subject, and MPO-DNA levels could not be generated for 3 subjects. Statistical analysis was performed using Kruskal-Wallis test and Dunn's multiple comparisons tests of severe/critical COVID-19 subjects according to dexamethasone treatment and ventilation.

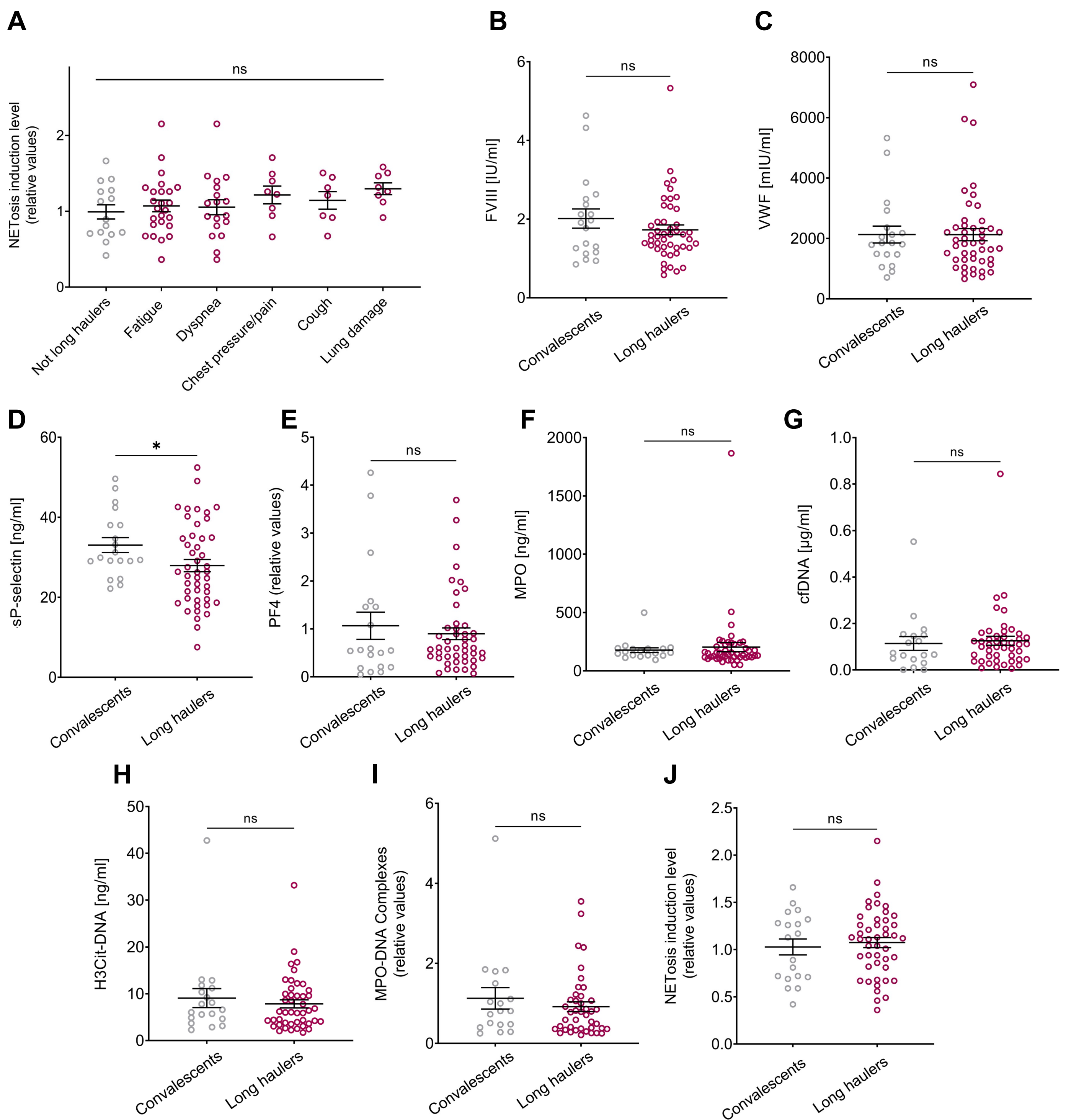

Figure S7: Platelet activation, coagulation factors and MPO-DNA complex levels in convalescent and Long COVID patient plasma. (A) NETosis induction levels of fully recovered subjects (n=15) and long haulers experienced the most common symptoms: fatigue (n=25), shortness of breath (n=18), chest pressure/pain (n=8), cough (n=7) and lung damage (n=8). No significant differences were found between the presented groups according to Kruskal-Wallis test (P = 0.2530). Comparisons between long haulers and all other convalescents were analyzed for **(B)** FVIII, convalescents n=19, long haulers n=46, P = 0.4288, **(C)** VWF, convalescents n=19, long haulers n=46, P = 0.9345, **(D)** sP-selectin, convalescents n=19, long haulers n=46, P = 0.0416, **(E)** PF4, convalescents n=19, long haulers n=46, P = 0.7776, **(F)** MPO convalescents n=18, long haulers n=46, P = 0.4213, **(G)** cfDNA convalescents n=18, long haulers n=46, P = 0.5812, **(H)** H3Cit-DNA, convalescents n=19, long haulers n=45, P = 0.5976, **(I)** MPO-DNA complex levels, convalescents n=18, long haulers n=42, P = 0.8666 and **(J)** NETosis induction level convalescents n=20, long haulers n=45, P = 0.9410. Statistical analysis was performed using Mann-Whitney tests.