

Supplementary materials

In-vitro NET-osis induced by COVID-19 sera is associated to severe clinical course in not vaccinated patients and immune-dysregulation in breakthrough infection

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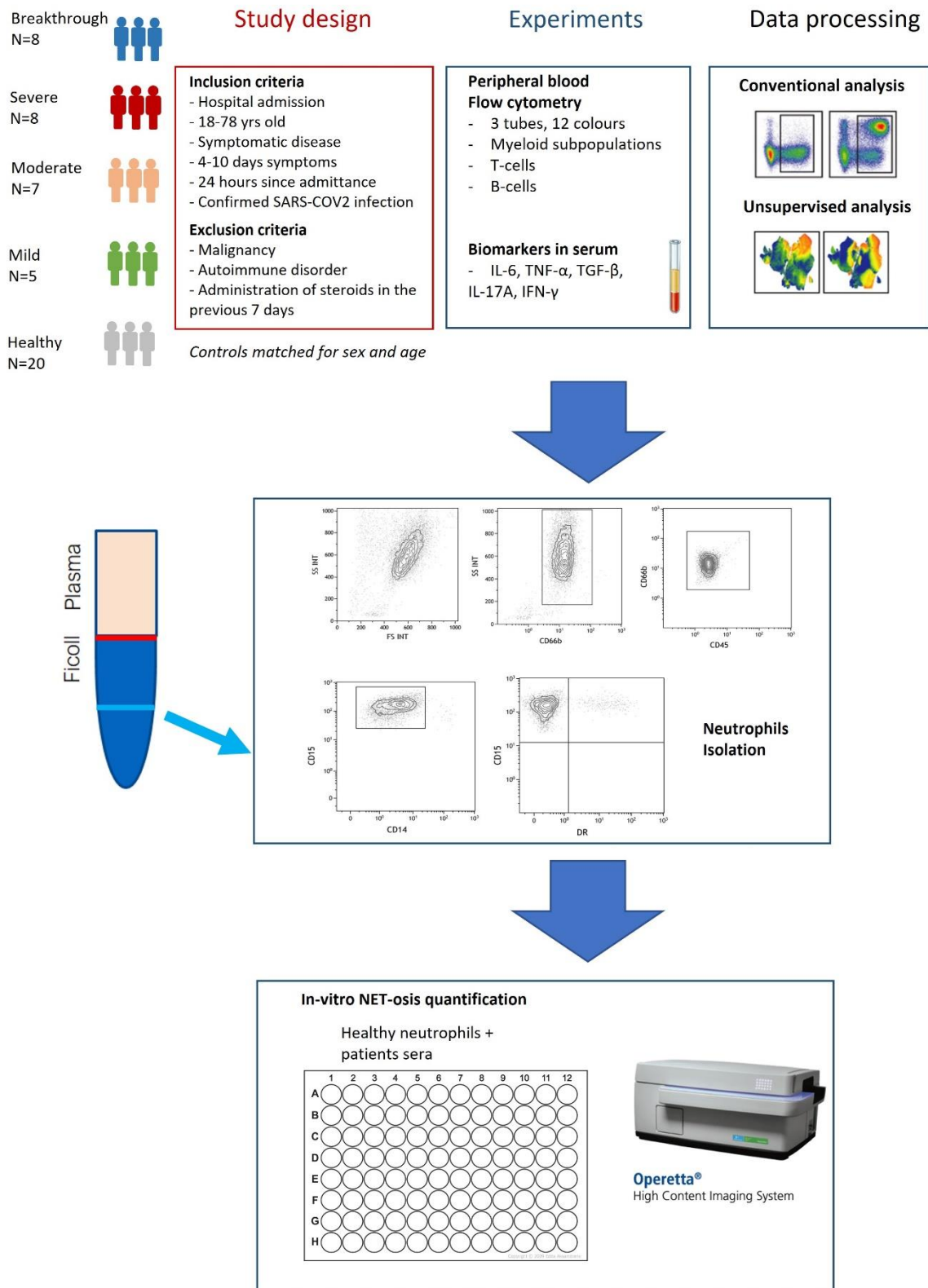
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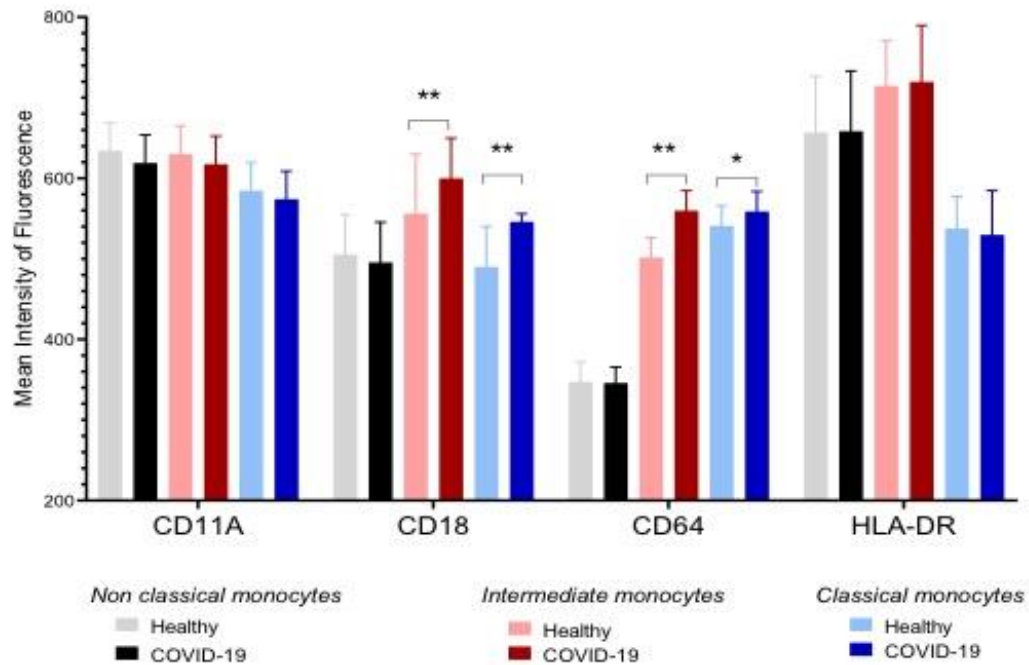
Supplementary Figure 1

Study design, inclusion criteria and flowchart depicting the overall experimental design of this study. Neutrophils were isolated from peripheral blood of healthy donors using a density gradient as described in the methods section, and the proportion of CD66b+ cells was calculated among CD15+ cells by flow cytometry. Three hours later neutrophils were incubated with sera obtained from healthy or COVID-19 subjects to quantify in-vitro NET-osis.



Supplementary Figure 2

Expression of activation markers CD11A, CD18, CD64 and HLA-DR in non-classical, intermediate and classical monocytes



	Non classical monocytes			Intermediate monocytes			Classical monocytes		
	Healthy	COVID	p-value	Healthy	COVID	p-value	Healthy	COVID	p-value
CD11A MFI	633.88	618.77	0.32	630.00	617.41	0.09	584.46	573.92	0.28
CD18 MFI	505.25	495.50	0.08	556.00	599.80	0.04	490.09	545.88	0.03
CD64 MFI	347.38	345.75	0.32	501.50	559.67	0.03	540.78	558.62	0.04
HLA-DR MFI	656.50	658.25	0.32	714.67	719.64	0.33	537.26	529.66	0.19

Supplementary Table 1

Statistical significance of the correlation matrix and heatmap of Pearson's r correlation coefficients among the multiple variables included in the analysis of COVID-19 patients shown in Figure 1

	MFI CD64 monocytes	MFI CD11A monocytes	MFI CD18 monocytes	MFI HLA-DR monocytes	MFI CD64 neutrophils	MFI CD16 neutrophils	T/mmc	T4/mmc	T4RA+/mmc	T4RO+/mmc	T8/mmc	mo_MDSC/mmc	T8RA+/mmc	T8RO+/mmc	IL-1-β (pg/mL)	TNF-α (pg/mL)	TGF-β (pg/mL)	IFN-γ (pg/mL)	IL-6 (pg/mL)	IL-17A (pg/mL)
MFI CD64 monocytes		0,00	0,231	0,195	0,557	0,380	0,781	0,896	0,227	0,484	0,977	0,139	0,504	0,146	0,079	0,831	0,755	0,094	0,180	0,577
MFI CD11A monocytes	0,00		0,072	0,02	0,083	0,03	0,956	0,827	0,564	0,636	0,654	0,461	0,537	0,05	0,344	0,415	0,244	0,107	0,080	0,180
MFI CD18 monocytes	0,231	0,072		0,235	0,480	0,135	0,346	0,558	0,606	0,210	0,239	0,697	0,299	0,895	0,996	0,576	0,566	0,861	0,684	0,563
MFI HLA-DR monocytes	0,195	0,02	0,235		0,434	0,03	0,368	0,910	0,173	0,787	0,331	0,610	0,127	0,906	0,442	0,405	0,732	0,899	0,508	0,430
MFI CD64 neutrophils	0,557	0,083	0,480	0,434		0,057	0,580	0,795	0,487	0,915	0,637	0,465	0,249	0,02	0,687	0,645	0,121	0,03	0,00	0,00
MFI CD16 neutrophils	0,380	0,03	0,135	0,03	0,057		0,160	0,698	0,310	0,156	0,346	0,799	0,513	0,207	0,133	0,647	0,083	0,163	0,217	0,083
T/mmc	0,781	0,956	0,346	0,368	0,580	0,160		0,538	0,01	0,179	0,00	0,082	0,01	0,204	0,970	0,124	0,153	0,507	0,04	0,184
T4/mmc	0,896	0,827	0,558	0,910	0,795	0,698	0,538		0,891	0,261	0,733	0,757	0,921	0,170	0,859	0,570	0,182	0,864	0,731	0,660
T4RA+/mmc	0,227	0,564	0,606	0,173	0,487	0,310	0,01	0,891		0,256	0,071	0,01	0,00	0,234	0,892	0,085	0,05	0,231	0,04	0,115
T4RO+/mmc	0,484	0,636	0,210	0,787	0,915	0,156	0,179	0,261	0,256		0,092	0,03	0,578	0,215	0,135	0,841	0,00	0,880	0,333	0,055
T8/mmc	0,977	0,654	0,239	0,331	0,637	0,346	0,00	0,733	0,071	0,092		0,054	0,065	0,367	0,963	0,831	0,479	0,721	0,04	0,302
mo_MDSC/mmc	0,139	0,461	0,697	0,610	0,465	0,799	0,082	0,757	0,01	0,03	0,054		0,108	0,761	0,405	0,505	0,268	0,498	0,02	0,616
T8RA+/mmc	0,504	0,537	0,299	0,127	0,249	0,513	0,01	0,921	0,00	0,578	0,065	0,108		0,064	0,538	0,02	0,070	0,072	0,02	0,02
T8RO+/mmc	0,146	0,05	0,895	0,906	0,02	0,207	0,204	0,170	0,234	0,215	0,367	0,761	0,064		0,540	0,332	0,00	0,088	0,00	0,00
IL-1-β (pg/mL)	0,079	0,344	0,996	0,442	0,687	0,133	0,970	0,859	0,892	0,135	0,963	0,405	0,538	0,540		0,288	0,259	0,644	0,468	0,433
TNF-α (pg/mL)	0,831	0,415	0,576	0,405	0,645	0,647	0,124	0,570	0,085	0,841	0,831	0,505	0,02	0,332	0,288		0,138	0,238	0,244	0,318
TGF-β (pg/mL)	0,755	0,244	0,566	0,732	0,121	0,083	0,153	0,182	0,05	0,00	0,479	0,268	0,070	0,00	0,259	0,138		0,207	0,04	0,00
IFN-γ (pg/mL)	0,094	0,107	0,861	0,899	0,03	0,163	0,507	0,864	0,231	0,880	0,721	0,498	0,072	0,088	0,644	0,238	0,207		0,00	0,088
IL-6 (pg/mL)	0,180	0,080	0,684	0,508	0,00	0,217	0,04	0,731	0,04	0,333	0,84	0,02	0,02	0,00	0,468	0,244	0,04	0,00		0,01
IL-17A (pg/mL)	0,577	0,180	0,563	0,430	0,00	0,083	0,184	0,660	0,115	0,055	0,302	0,616	0,02	0,00	0,433	0,318	0,00	0,088	0,01	

Supplementary Table 2

Concentration of cytokines and absolute counts of immune cells in peripheral blood in patients who achieved viral clearance by 28 days from hospital admission

		Viral clearance by 28 days		p-value ^a
		Yes (N=7)	No (N=13)	
IL1-β	Mean (pg/mL)	65.3	86.1	<i>0.03</i>
	SD	6.9	6.1	
TNF-α	Mean (pg/mL)	7.4	10.9	<i>0.007</i>
	SD	0.3	0.8	
TGF-β	Mean (pg/mL)	5159	15392	0.003
	SD	867	1835	
IFN-γ	Mean (pg/mL)	193.1	258.8	0.01
	SD	1.5	24.7	
IL-6	Mean (pg/mL)	3.8	123.5	0.02
	SD	1.1	49.9	
IL-17A	Mean (pg/mL)	30.6	89.2	<0.0001
	SD	8.2	1.4	
NK-cells	Mean (cells/mmc)	299	150	0.01
	SD	58	26	
CD8⁺ T cells	Mean (cells/mmc)	533	292	0.007
	SD	65	47	
CD4⁺ T cells	Mean (cells/mmc)	1007	532	0.0005
	SD	67	75	
CD4⁺CD45RO⁺ T-cells	Mean (cells/mmc)	598	343	0.003
	SD	53	47	
CD14⁺HLA-DR⁺ monocytes	Mean (cells/mmc)	17	56	0.01
	SD	2	12	
CD14⁺CD16⁺ monocytes	Mean (cells/mmc)	38	86	0.01
	SD	5	15	

^a based on T-Student tests; p-values <0.05 are indicated in bold font.
Results are reported as mean and standard deviation (SD)

Supplementary Table 3 Descriptive statistics of immunological classifications

Characteristics	Frequency (%)
Inflammatory monocytes%	
Low	9 (45%)
High	11 (55%)
moMDSC%	
Low	11 (55%)
High	9 (45%)
CD4/CD45RA%	
Low	15 (75%)
High	5 (5%)
CD4/CD45R0%	
Low	3 (15%)
High	17 (85%)

Supplementary Table 4 Comparison of clinical characteristics and treatments according to immunological classifications

Characteristics	Inflammatory monocytes %			moMDSC%			CD4+CD45RA+%			CD4/CD45RO%		
	Low	High	p-value ^a	Low	High	p-value ^a	Low	High	p-value ^a	Low	High	p-value ^a
Age, years	53.0 (13.3)	68.9 (11.9)	0.011	62.2 (14.4)	61.2 (15.7)	0.891	64.7 (14.6)	58.8 (11.5)	0.118	50.9 (16.0)	63.4 (14.0)	0.168
Comorbidities	77.80%	100%	0.099	100%	77.80%	0.099	85.70%	92.30%	0.639	66.70%	94.10%	0.144
Severity illness at admission												
Mild	33.30%	18.20%	0.522	18.20%	33.30%	0.723	28.60%	23.10%	0.339	33.30%	23.50%	0.375
Moderate	22.20%	45.50%		35.40%	33.30%		14.30%	46.20%		0.00%	100.00%	
Severe	44.40%	36.40%		45.50%	33.30%		57.10%	30.80%		66.70%	35.30%	
Pre-infection administration of drugs	77.80%	72.70%	0.795	81.80%	66.70%	0.436	85.70%	69.20%	0.417	66.70%	7.60%	0.718
Post-infection administration of steroids	66.70%	36.40%	0.178	54.50%	44.40%	0.653	71.40%	38.50%	0.16	66.70%	47.10%	0.531
Heparin administration	44.40%	63.60%	0.391	54.50%	55.60%	0.964	28.60%	69.20%	0.081	0.00%	64.70%	0.038

^a based-on Chi-squared or T-Student tests; p-values <0.05 are indicated in bold font
Results are reported as mean (SD) or percentage

Supplementary Table 5 Main outcomes according to immunological classification

Immunological classification		Pneumonia or more severe conditions*	Hospitalized or died at 28 days*	Time To Viral Clearance (days)*
Inflammatory monocytes %	Low	63.6%	11.1%	23.7 (13.4)
	High	77.8%	9.1%	36.4 (15.6)
	p-value ^a	0.492	0.999	0.126
moMDSC%	Low	77.8%	0%	32.4 (10.8)
	High	63.6%	22.2%	29.2 (24.3)
	p-value ^a	0.492	0.189	0.724
CD4/CD45RA%	Low	60.0%	13.3%	31.4 (16.8)
	High	73.3%	0%	31.3 (14.5)
	p-value ^a	0.573	0.999	0.991
CD4/CD45R0%	Low	70.6%	0%	20.0 (12.5)
	High	66.7%	11.8%	34.2 (15.5)
	p-value ^a	0.891	0.999	0.170

*based on Chi-squared, Fisher Exact, or T-Student tests; results are reported as mean (SD) or percentage

Supplementary Table 6

Descriptive statistics of the study population at baseline of their COVID-19 diagnosis

Characteristics	Mild-moderate COVID 19 not vaccinated (group 1, N=12)	Breakthrough COVID19 (group 2, N=21)	p-value ^a
Median age, years (range)	54 (41-77)	46 (29-63)	0.07
Males, N (%)	5 (41)	10 (48)	0.70
Two or more comorbidities at admission, N (%)	10 (83)	0 (0)	<0.0001
Diabetes	1 (8)	0 (0)	0.19
Hypertension	7 (58)	3 (14)	0.009
Chronic obstructive pulmonary disease	3 (25)	2 (9)	0.22
Hyperlipidemia	7 (58)	4 (19)	0.02
Pre-admission administration of drugs for comorbidities	7 (58)	0 (0)	0.82
Median Haemoglobin, g/dL (range)	12.8 (10.4-15.1)	14.3 (9.4-15.6)	0.06
Median Absolute white blood cells *10 ⁶ /mmc (range)	5.8 (3.6-7.0)	6.6 (3.4-12.3)	0.19
Median Absolute neutrophils *10 ⁶ /mmc (range)	3.8 (3.3-5.1)	4.2 (2.8-6.1)	0.13
Median Absolute lymphocytes *10 ⁶ /mmc (range)	1.6 (0.9-2.2)	1.8 (0.6-2.8)	0.83
Median Absolute monocytes *10 ⁶ /mmc (range)	0.5 (0.3-0.6)	0.4 (0.2-0.6)	0.96
Median Absolute platelets *10 ⁹ /mmc (range)	218 (152-260)	242 (89-410)	0.92
Median LDH, UI/L (range)	223 (136-300)	156 (120-332)	0.04
Median Reactive C-protein, UI/L (range)	6.1 (1.4-22.3)	2.3 (1.8-3.9)	0.01
Median IgA, g/dL (range)	300 (210-368)	290 (240-360)	0.96
Median IgM, g/dL (range)	72 (70-123)	96 (85-132)	0.61
Median IgG, g/dL (range)	1050 (930-1376)	1086 (870-1400)	0.66
Median D-dimer, ng/mL (range)	245 (88-524)	30 (8-16)	0.02
Median anti-SARS-CoV-2 antibodies (IgG) titer, BAU (range)	0 (NA)	75.2 (4.0-135.0)	NA

^a based on ANOVA test for the comparison of findings in COVID-19 patients; p-values <0.05 are indicated in bold font. Results are reported as median and interquartile (IQ) range.