Distinct cytokine profiles associated with COVID-19 severity and mortality

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Methods

Cytokine levels measurement by immunoassays

Whole blood was collected in anticoagulant free tubes and serum was separated by centrifugation and stored at -80°C less than two hours after sampling

Concentrations of IL-1β, IFN-γ, IL-6, IL-8, IL-22, TNF-α and IL-10 were determined using a seven-plex planar array immunoassay on the Quanterix® SP-X™ imaging and analysis platform with reagents and procedures obtained from Quanterix Corporation (Quanterix Human CorPlex Cytokine Panel Array, Lexington, MA, USA). Briefly, each well of a 96-well microplate was pre-spotted with analyte-specific capture antibodies (Abs) and incubated for 2 hours with 4x diluted serum or calibrators at room temperature and were swirled at 225 rpm. After washing, a mixture of biotinylated analyte-specific detection Abs was added and plates were incubated for 30 minutes. After washing, streptavidin-horseradish peroxidase was added for 30 minutes. After the last wash, a mixture of luminol and peroxide solution was added into the plate to produce a luminescent signal, detected by the SP-X Imaging System, resulting in a signal intensity directly proportional to the quantity of each analyte in the standard or sample of interest. Calibrators were run in duplicate and fit with a five-parameter logistic (5PL) regression.

The Simoa™ (single molecule array) HD-1 analyzer (Quanterix, Lexington, MA, USA) was used for ultrasensitive immunodetection (digital ELISA) of IL-17A, IL-18, GM-CSF and IFN-α, using single-plex bead-based assays, according to the manufacturer's instructions.

Calibrators were run in duplicate and fit with a four-parameter logistic (4PL) regression, with $1/y^2$ weighting.

Serum IFN-β levels were quantified using a highly sensitive ELISA kit (PBL Assay Science, Piscataway, NJ, USA) based on a two-step assay, according to the manufacturer's instructions. Calibrators were run in duplicate and fit with a four-parameter logistic (4PL) regression.

The concentration of each cytokine in unknown samples was interpolated from the calibration curve by multiplying by the dilution factor. All cytokine concentrations were expressed in pg/mL. Samples with non-detectable values were replaced by the limit of detection value (LOD), while those over the detection range were replaced by the upper limit of quantification (ULOQ) (see details in Table E3).

N-antigen level assessment

Serum nucleocapsid (N) antigen levels were determined with the COV-QUANTO® ELISA kit (AAZ, Boulogne Billancourt, France), according to manufacturer's recommendations. In each plate, 5 calibrators were run to quantify the concentration of N-Antigen in the patient's serum. Samples with undetectable levels were replaced by half of the LOD value (2.97 pg/mL), while those over the detection range were replaced by 1.5 x the ULOQ value (180 pg/mL).

PCA analysis and construction of prediction functions

Identification of the cytokine combinations associated with severity and mortality was guided by unsupervised principal component analysis (PCA), performed using R v3.6.2 with the FactoExtra, ggbiplot and prcomp functions, on z-scaled log10-transformed cytokine concentrations. Samples with missing data were excluded from the PCA analysis resulting in

somewhat variable numbers of patients analyzed. PCA analysis started with twelve measured cytokines. Then, the variables with no contribution to inter-patient variation and/or to the separation of the severity or mortality groups were excluded. The statistical significance of the separation between severity and mortality groups within the PCA was assessed by the non-parametric Fisher-Exact test based on the numbers of patients from each group above/below (or inside/outside) the separatrix line (square) for 1 (or 2) dimensional separation.

In order to obtain prediction or classification functions that were realistic to measure, we identified the cytokine combinations that needed the smallest number of cytokines using only log-transformed cytokine concentrations without z-scaling. This was achieved objectively and systematically by sorting the cytokine concentrations according to their largest absolute PC factor value (either from only the relevant PC1 or PC2 for one-dimensional separation, or from both PC1 and PC2 for two-dimensional separation) in each relevant PCA, and then by selecting the minimum number of cytokines as log10 concentrations (factorized by the corresponding PC factor) showing a statistically significant difference, using the Mann-Whitney non-parametric test between the relevant patient groups. The prediction and classification functions obtained were:

<u>For severity classification:</u>

fcc-INFLAM = 0.453*Log[TNF-α] + 0.444*Log[IL-6] + 0.426*Log[IL-8] + 0.426*Log[IL-10]

fcc-IFNI = 0.702*Log[IFN-α] + 0.689*Log[IFN-β]

<u>For mortality prediction in No-MVS group:</u>

$f_{NO\text{-}MVS}$ = 0.475*Log[IFN-α] + 0.188*Log[IFN-β]

<u>For mortality prediction in MVS (SAPS-II ≥ 35) group:</u>

f_{MVS} = 0.474*Log[TNF-α] + 0.444*Log[IL-10] + 0.194*Log[IFN-α]

Of note, for the MVS group with SAPS-II ≥ 35, the surviving patients are separated two dimensionally in the 3rd quartile. Therefore, we used PC factors based on the transformation of PC1 and PC2 into a vector with a -135° angle.

For mortality prediction in ECMO group:

f_{ECMO} = 0.414*Log[IL-10] - 0.609*Log[IL-17A] - 0.352*Log[IL-18]

Of note, the factors for IL-17A and IL-18 in f_{ECMO} are negative because they are negatively associated with mortality.

Lastly, to obtain the mortality prediction tables (true-negative, true-positive, false-negative and false-positive) and associated prediction scores (accuracy, specificity, sensitivity, negative-predictive-value and positive-predictive-value, Risk Ratio), a threshold was determined on the respective prediction functions with the highest accuracy:

No-MVS: $f_{NO\text{-}MVS} > 0.59$

MVS (SAPS-II ≥ 35): $f_{MVS} > 0.5$

ECMO: $f_{ECMO} > 0.1$

These thresholds were subsequently tested for statistical significance by the Fisher-exact test.

Table E1. **Demographics, baseline characteristics and clinical outcomes of 115 COVID-19 patients in the initial cohort (initial cohort).**

	No mechanical ventilatory support (N=34)	**Mechanical ventilatory support (N=50)**	**ECMO support (N=31)**	**All patients (N=115)**	**p values¥**
Median age – yr (IQR)	73 (46 – 73)	63 (55 – 69)	49.5 (42 – 56)	58 (49 – 66)	**< 0.001**
Male sex – no. (%)	22 (64.7)	36 (72)	25 (80.6)	83 (72.2)	ns
Severity score at baseline					
SAPS II – median (IQR)	26 (18 – 33)	35 (27 – 44)	52 (45 – 65)	36 (26 – 49)	**< 0.001**
SOFA – median (IQR)	-	7 (4 – 7)	12 (9 – 15)	-	**< 0.001**
Respiratory severity					
Nasal cannula or high concentration mask	25 (73.5)	-	-	25 (21.7)	
Non-invasive ventilation or high-flow nasal cannula	-	10 (20)	-	10 (8.7)	
Invasive mechanical ventilation	-	40 (80)	31 (100)	71 (61.7)	
Extracorporeal membrane oxygenation	-	0 (0)	31 (100)	31 (27)	
Time from onset of symptoms to admission					
Median days – no. (IQR)	8 (2 – 11)	9 (7 – 11)	12 (8 – 15)	9 (6 – 12)	**0.001**
Past medical history – no. (%)					
Cardiovascular disease	11 (32.4)	10 (20)	2 (6.5)	23 (20)	ns
Type 2 diabetes	11 (37.4)	19 (39.6)	12 (38.7)	42 (36.5)	ns
Body mass index (kg/m^2)	*NA*	26.7	30.5		
Normal (18.5-25)	15 (44.1)	17 (34)	2 (6.5)	34 (29.6)	**0.002**
Overweight (25-30)	12 (35.3)	15 (30)	10 (32.3)	37 (32.2)	ns
Obesity (≥30)	7 (20.6)	18 (36)	19 (61.3)	44 (38.3)	**0.03**
Hypertension	19 (55.9)	29 (58)	17 (54.8)	65 (56.5)	ns
Immunocompromised*	3 (8.8)	6 (12)	1 (3.3)	10 (8.7)	ns
Malignant tumour	5 (14.7)	3 (6)	0 (0)	8 (7)	ns
Chronic neurologic disease	5 (14.7)	1 (2)	0 (0)	6 (5.2)	**0.01**
Chronic pulmonary disease	6 (17.6)	8 (16)	4 (12.9)	18 (15.7)	ns
Chronic kidney disease	6 (17.6)	11 (22)	1 (3.2)	18 (15.7)	ns
Chronic liver disease	2 (5.9)	1 (2)	0 (0)	3 (2.6)	ns
Smoking habits					
Never smoker	21 (61.8)	43 (86)	28 (90.3)	92 (80)	**0.006**
Former smoker	11 (32.4)	6 (12)	3 (9.7)	20 (17.4)	**0.02**
Daily smoker	2 (5.9)	1 (2)	0 (0)	3 (2.6)	ns
Past history of arterial or venous thrombosis					
Arterial	2 (5.9)	5 (10)	1 (3.2)	8 (7)	ns
Venous	3 (8.8)	0 (0)	0 (0)	3 (2.6)	**0.03**
Treatment regimen at baseline – no. (%)					
Long-term immunosuppressive agent use	5 (14.7)	7 (14)	1 (3.2)	13 (11.3)	ns
Glucocorticoids	8 (23.5)	5 (10)	1 (3.2)	14 (12.2)	**0.04**
Recent chemotherapy for cancer	3 (8.8)	0 (0)	0 (0)	3 (2.6)	**0.03**
Angiotensin converting enzyme inhibitor	6 (17.7)	5 (10)	4 (12.9)	15 (13)	ns

Angiotensin II receptor blockers	6 (17.7)	12 (24)	4 (12.9)	22 (19.1)	ns
Median laboratory values at baseline (IQR)					
Neutrophil count – $x10^9$/L [normal range : 2.7 – 5]	3.45 (2.86 – 5.55)	7.38 (3.9 – 10.7)	11.6 (8.76 – 14)	7.64 (3.7 – 11.5)	**< 0.001**
Lymphocyte count – $x10^9$/L [normal range: 1.5 – 4]	0.99 (0.78 – 1.31)	0.81 (0.56 – 1.08)	0.86 (0.64 – 1.08)	0.88 (0.6 – 1.14)	**0.04**
Platelet count – $x10^9$/L [normal range: 150 – 400]	180 (146 – 246)	184 (134 – 254)	251 (199 – 302)	196 (146 – 272)	**0.01**
Lactate dehydrogenase – U/L [normal range: 135-215]	351 (295 – 486)	560 (490 – 683)	590 (436 – 822)	508 (398 – 678)	**< 0.001**
Serum ferritin – µg/L [normal range: 15-150]	817 (355 – 1312)	1743 (984 – 2480)	2007 (1383 – 3145)	1466 (845 – 2465)	**< 0.001**
Treatment – no. (%)					
Hydroxychloroquine	7 (20.6)	27 (54)	18 (58.1)	52 (45.2)	**0.003**
Glucocorticoids	4 (11.8)	1 (2)	9 (29)	14 (12.2)	**0.002**
Tocilizumab or sarilumab	0 (0)	7 (14)	3 (9.7)	10 (8.7)	0.08
Oseltamivir	0 (0)	9 (18)	2 (6.5)	11 (9.6)	**0.02**
Remdesivir	0 (0)	2 (4)	2 (6.5)	4 (3.5)	0.36
Antibiotic therapy	28 (82.4)	45 (90)	31 (100)	104 (90.4)	**0.04**
Hemodialysis	0 (0)	8 (16)	13 (41.9)	21 (18.3)	
Complications – no. (%)					
Acute respiratory distress syndrome	2 (5.9)	43 (86)	31 (100)	76 (66.1)	**< 0.001**
Acute kidney injury	8 (23.5)	22 (44)	18 (58.1)	48 (41.7)	**0.02**
Ventilator associated pneumonia	0 (0)	13 (26)	29 (93.6)	42 (36.5)	**< 0.001**
Shock	0 (0)	17 (34)	19 (61.3)	36 (31.3)	**< 0.001**
Pulmonary embolism	1 (2.9)	4 (8)	8 (25.8)	13 (11.3)	**0.01**
Thrombosis					
Venous	0 (0)	5 (10)	18 (58.1)	23 (20)	**< 0.001**
Arterial	0 (0)	3 (6)	0 (0)	3 (2.6)	ns
Clinical outcome at day 30 – no. (%)					
Discharged	27 (79.4)	30 (60)	20 (64.5)	77 (67)	ns
Remained in hospital	0 (0)	1 (2)	4 (12.9)	5 (4.3)	ns
Deceased	7 (20.6)	19 (38)	7 (22.6)[£]	33 (28.7)[£]	ns
Median length of stay[Ω], days (IQR)	10 (6 – 16)	10 (7 – 22)	32 (25 – 55)	13 (7 – 29)	**< 0.001**
Causes of death[♠]	7 patients	19 patients	7 patients	33 patients	
ARDS	0 (0)	9 (47.4)	3 (42.9)	12 (36.4)	
Respiratory failure	5 (71.4)	1 (5.3)	0 (0)	6 (18.2)	
Septic shock	2 (28.6)	1 (5.3)	5 (71.4)	8 (24.2)	
Multiple organ failure	0 (0)	3 (15.8)	3 (42.9)	6 (18.2)	

[¥] Kruskal-Wallis test for continuous variables, Fisher exact test for discrete variables, bold values indicate statistical significance.
*including cardiac, liver or kidney allograft, hematopoietic stem cell transplantation, or immunosuppressive agent for auto-immune disease.
[£]2 additional patients died later than day 30 of hospitalisation (at day 46 and day 50).
[Ω]As of June 18, 2020.
[♠]Some causes could not be assessed, and some are associated
ECMO, Extracorporeal Membrane Oxygenation; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment.

Table E2. **Demographics, baseline characteristics and clinical outcomes of the 86 COVID-19 patients in the validation cohort.**

	No mechanical ventilatory support (N=10)	**Mechanical ventilatory support (N=58)**	**ECMO support (N=18)**	**All patients (N=86)**	**p value¥**
Median age – yr (IQR)	65 (54 – 73)	66 (56 – 72)	52 (43 – 61)	64 (52 – 71)	ns
Male sex – no. (%)	5 (50)	40 (69)	11 (61.1)	56 (65.1)	ns
Severity score at baseline					
SAPS II – median (IQR)	23 (18 – 27)	36 (28 – 48)	56 (43 – 61)	37 (27 – 56)	**0.02**
Respiratory severity					
None	3 (30)	-	-	3 (3.5)	
Nasal cannula or high concentration mask	7 (70)	1 (1.7)	-	8 (9.3)	
Non-invasive ventilation or high-flow nasal cannula	-	12 (20.7)	-	12 (14)	
Invasive mechanical ventilation	-	45 (77.6)	18 (100)	63 (73.3)	
Extracorporeal membrane oxygenation	-	0 (0)	18 (100)	18 (20.9)	
Time from onset of symptoms to day of sample					
Median days – no. (IQR)	13 (7 – 14)	12 (10 – 14)	15 (11 – 19)	12 (10 – 15)	0.07
Past medical history – no. (%)					
Cardiovascular disease	2 (20)	9 (15.5)	0 (0)	11 (12.8)	ns
Type 2 diabetes	2 (20)	11 (19)	7 (38.9)	20 (23.6)	ns
Body mass index (median, kg/m^2)	24.2 (22.7 – 27.1)	29.4 (25.7 – 33.5)	33.1 (30.7 – 38.7)	30.2 (25.7 – 34.7)	ns
Normal (18.5-25)	5 (50)	14 (24.6)	2 (11.1)	21 (25)	**0.04**
Overweight (25-30)	3 (30)	16 (28.1)	0 (0)	19 (22.6)	**0.01**
Obesity (≥30)	1 (10)	27 (47.4)	16 (88.9)	44 (52.4)	**0.0001**
Hypertension	6 (60)	23 (39.7)	8 (44.4)	37 (43)	ns
Immunocompromised*	3 (30)	2 (3.5)	1 (5.6)	6 (7)	**0.02**
Malignant tumour	1 (10)	6 (10.3)	0 (0)	7 (8.1)	ns
Chronic neurologic disease	0 (0)	0 (0)	1 (5.6)	1 (1.2)	ns
Chronic pulmonary disease	0 (0)	20 (34.5)	3 (16.7)	23 (26.7)	**0.03**
Chronic kidney disease	1 (10)	5 (8.6)	3 (16.7)	9 (10.5)	ns
Chronic liver disease	2 (20)	0 (0)	0 (0)	2 (2.4)	**0.01**
Smoking habits					
Never smoker	1 (10)	35 (60.3)	16 (88.9)	59 (68.6)	ns
Former smoker	1 (10)	20 (34.5)	2 (11.1)	23 (26.7)	ns
Daily smoker	8 (80)	3 (5.2)	0 (0)	4 (4.7)	ns
Past history of arterial or venous thrombosis					
Arterial	0 (0)	1 (1.7)	0 (0)	1 (1.2)	ns
Venous	0 (0)	2 (3.5)	0 (0)	2 (2.3)	ns
Treatment regimen at baseline – no. (%)					
Long-term immunosuppressive agent use	3 (30)	4 (6.9)	1 (5.6)	8 (9.3)	ns
Glucocorticoids	3 (30)	3 (5.2)	1 (5.6)	7 (8.1)	ns
Recent chemotherapy for cancer	0 (0)	1 (1.7)	0 (0)	1 (1.2)	ns
Angiotensin converting enzyme inhibitor	1 (10)	8 (13.8)	1 (5.6)	10 (11.6)	ns
Angiotensin II receptor blockers	1 (10)	6 (10.3)	5 (27.8)	12 (14)	ns
Median laboratory values at baseline (IQR)					

Neutrophil count – x10^9/L [normal range : 2.7 – 5]	6.0 (3.2 – 8.8)	8.7 (4.7 – 10)	12.2 (8.4 – 19.4)	8.7 (4.7 – 11.9)	ns
Lymphocyte count – x10^9/L [normal range: 1.5 – 4]	0.8 (0.6 – 1.3)	1.2 (0.8 – 1.4)	1.0 (0.6 – 1.3)	1.0 (0.7 – 1.3)	ns
Lactate dehydrogenase – U/L [normal range: 135-215]	381 (263 – 429)	446 (378 – 536)	467 (425 – 662)	430 (351 – 531)	ns
Serum ferritin – µg/L [normal range: 15-150]	648 (319 – 1092)	1007 (546 – 1805)	1180 (700 – 1749)	919 (648 – 4085)	ns
Treatment – no. (%)					
Hydroxychloroquine	0 (0)	0 (0)	0 (0)	0 (0)	ns
Glucocorticoids	9 (90)	58 (100)	18 (100)	85 (98.8)	ns
Tocilizumab or sarilumab	0 (0)	2 (3.5)	0 (0)	2 (2.3)	ns
Remdesivir	0 (0)	0 (0)	1 (5.6)	1 (1.2)	ns
Antibiotic therapy	5 (50)	39 (69.6)	18 (100)	62 (73.8)	**0.002**
Hemodialysis	0 (0)	6 (10.5)	7 (38.9)	13 (15.3)	**0.008**
Complications – no. (%)					
Acute respiratory distress syndrome	0 (0)	53 (91.4)	18 (100)	70 (81.4)	**< 0.0001**
Acute kidney injury	3 (30)	22 (38.6)	10 (55.6)	35 (41.2)	ns
Ventilator associated pneumonia	0 (0)	31 (56.4)	18 (100)	49 (59)	**< 0.0001**
Shock	0 (0)	14 (25.5)	10 (55.6)	24 (28.9)	**0.004**
Pulmonary embolism	0 (0)	1 (1.7)	2 (14.3)	3 (6.8)	ns
Thrombosis					
Venous	0 (0)	1 (8.3)	4 (22.2)	5 (5.8)	**0.01**
Arterial	0 (0)	1 (1.7)	3 (16.7)	4 (4.7)	**0.02**
Clinical outcome at 1 month– no. (%)					
Discharged	10 (100)	42 (72.4)	12 (66.6)	64 (74.4)	**0.03**
Remained in hospital	0 (0)	0 (0)	2 (11.1)	2 (2.3)	ns
Deceased[£]	0 (0)	16 (27.6)	4 (22.2)	20 (23.3)	ns
Median length of stay[Ω], days (IQR)	12 (8 – 14)	25 (15 – 35)	32 (18 – 46)	22 (14 – 67)	ns
Causes of death[♠] - no. (%)	0 patient	16 patients	4 patients	20 patients	
ARDS	--	2 (12.5)	1 (25)	3 (15)	
Septic shock	--	1 (6.25)	0 (0)	1 (5)	
Multiple organ failure	--	2 (12.5)	2 (50)	4 (20)	
Cardiac arrest	--	0 (0)	1 (25)	1 (5)	
No data	--	11 (68.75)	0 (0)	11 (55)	

[¥] Kruskal-Wallis test for continuous variables, Fisher exact test for discrete variables, bold values indicate statistical significance.
*Including cardiac, liver or kidney allograft, hematopoietic stem cell transplantation, or immunosuppressive agent for auto-immune disease.
[£]Four additional patients died later than day 30.
[Ω]As of January 30, 2021.
[♠]Cause of death data available only for 9 patients.
ECMO, Extracorporeal Membrane Oxygenation; SAPS II, Simplified Acute Physiology Score II.

Table E3. **Immunoassay specificity.**

Assays	Calibration range (pg/mL)	Serum dilution factor	LOD (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
Human CorPlex™ Cytokine Panel 7-Plex array, Quanterix, # 85-0410					
IL-1β	0.073-100	4	0.011	0.10	400
IFN-γ	0.012-50	4	0.007	0.05	200
IL-6	0.073-300	4	0.037	0.59	1200
IL-8	0.098-400	4	0.115	1.56	1600
IL-22	0.024-100	4	0.010	0.10	400
TNF-α	0.098-400	4	0.063	0.39	1600
IL-10	0.024-100	4	0.012	0.10	400
Simoa™ IL-17A Advantage Kit, Quanterix, #101599	0.041-30	4	0.017	0.084	120
Simoa™ IL-18 Discovery Kit, Quanterix, #102700	0.011-45	50	0.200	0.600	2250
Simoa™ GM-CSF Advantage Kit, Quanterix, #102329	0.041-30	4	0,008	0.041	120
Simoa™ IFN-α Advantage Kit, Quanterix, #100860	0.028-27.3	2	0.016	0.064	54.6
VeriKine-HS™ Human IFN Beta ELISA Kit, PBL Assay Science, #41415	1.2-150	1	0.59	1.15	150

IFN: interferon, IL: interleukin; GM-CSF: granulocyte macrophage colony-stimulating factor; TNF-α: tumor necrosis factor α; LOD: limit of detection; LLOQ: lower limit of quantification; ULOQ: upper limit of quantification.

Table E4. **Comparison of the 3 mortality predictors per respiratory severity group using Relative Sensitivity (rSens) and Relative Specificity (rSpec)** between the corresponding cytokine combination function predictor ($f_{No\text{-}MVS}$, f_{MVS} and f_{ECMO}) for each group accordingly versus the predictors for the other 2 groups.

rSens	Mortality predictor [1]		
Group	$f_{No\text{-}MVS}$	f_{MVS}	f_{ECMO}
No-MVS	--	1.64	>2.55
MVS*	0.96	--	0.98
ECMO	1.91	3.02	--

rSpec	Mortality predictor [1]		
Group	$f_{No\text{-}MVS}$	f_{MVS}	f_{ECMO}
No-MVS	--	1.00	1.27
MVS*	1.93	--	1.96
ECMO	1.24	1.25	--

rSens*rSpec	Mortality predictor [1]		
Group	$f_{No\text{-}MVS}$	f_{MVS}	f_{ECMO}
No-MVS	--	1.64	3.25
MVS*	1.86	--	1.92
ECMO	2.36	3.78	--

1) Cytokine combination function predictors of mortality as in Table 2:

No-MVS: $f_{NO\text{-}MVS} > 0.59$

MVS (SAPS-II ≥ 35): $f_{MVS} > 0.5$

ECMO: $f_{ECMO} > 0.1$

* MVS patients with SAPS-II ≥ 35, where SAPS-II =35 is the median for MVS patients.

Supplementary Figure Legends

Figure E1**: SAPS-II association with respiratory severity groups and mortality**. **A**) The patients' SAPS-II score was associated ($p<0.001$) with the respiratory severity (medians of 26, 35 and 52 for the No-MVS, MVS and ECMO groups, respectively). Overall, higher SAPS-II scores were associated with mortality (median 40.5 versus 32 for deceased versus surviving patients, $p=0.028$). A SAPS-II threshold of 35 (dotted line) gives the best SAPS-II prediction of mortality, albeit with an accuracy of only 65.1%, a sensitivity of 80.0% and a specificity of 59.2% (Risk Ratio=3.7, [95% CI 1.6-8.3], $p=0.0004$). Of note, the SAPS-II score was not associated with mortality in the No-MVS and ECMO groups, but only in the MVS group, where 4% (1/25) of the patients with SAPS-II <35 were deceased at one month, in contrast to 64% (16/25, $p<0.0001$) in the group of patients with a SAPS-II value ≥35. **B**) PCA of the 8 cytokines most contributing to inter-patient variation in COVID-19 patients (same as PCA in Figure 1B), annotated by severity groups classified according to SAPS-II scores (moderate: 0-26; severe: 27-52; critical: 53-89), shows distinct separation of patients according to the SAPS-II severity, independently of the oxygen support modality (No-MVS, MVS or ECMO). Statistical analysis was performed with Mann-Whitney U test: ns non-significant; ✱✱✱ $p<0.001$, ✱✱✱✱ $p<0.0001$.

SAPS II: simplified acute physiology score II; Dec.: deceased.

Figure E2: **Serum cytokine levels among healthy controls and COVID-19 patients, classified according to their respiratory severity and mortality.** Comparison of serum cytokine levels in COVID-19 patients (n=115), divided into groups without mechanical ventilatory support (no MVS, n=34, 27 alive and 7 deceased), with mechanical ventilatory support (MVS, n=50, 31 alive and 19 deceased), or with extracorporeal membrane oxygenation

(ECMO, n=31, 25 alive and 6 deceased). Serum samples from ten SARS-CoV-2-negative healthy donors were included as controls. Symbols represent individual samples; bars indicate median values. Statistical analyses were conducted using the Mann-Whitney U test (ns: non-significant; ✱ $p<0.05$; ✱✱ $p<0.01$; ✱✱✱ $p<0.001$; ✱✱✱✱ $p<0.0001$).

Ctrls: controls; Dec.: deceased; IFN: interferon; IL: interleukin; GM-CSF: granulocyte macrophage colony-stimulating factor; TNF-α: tumor necrosis factor α; ULOD: under limit of detection.

Figure E3**: Subgrouping of COVID-19 MVS patients in relation with cytokine combinations. A**) K-means clustering of the mechanical ventilation support (MVS) patients in the PCA from Figure 1B indicates that MVS patients can be split into three different ($p<0.01$) sub-groups based on their cytokine combination profile: MVS-1 group, showing low levels of fcc-$_{IFNI}$ and being similar to ECMO patients, MVS-2 showing high levels of fcc-$_{IFNI}$ similarly to No-MVS patients, and MVS-3 group expressing high levels of both fcc-$_{INFLAM}$ and fcc-$_{IFNI}$. **B-C)** Levels of the cytokine combination functions fcc-$_{INFLAM}$ and fcc-$_{IFNI}$, based on the most contributing factors in PC1 and PC2 accordingly, are depicted as function of the respiratory severity groups including the MVS subgrouping.

Ellipses in PCA represent the 68% confidence interval (CI) of patient distribution in each group.

Cytokine combination functions definitions are given in the supplementary information.

Statistical analysis was performed using the Mann-Whitney U test: ns non-significant; ✱ $p<0.05$; ✱✱ $p<0.01$; ✱✱✱ $p<0.001$; ✱✱✱✱ $p<0.0001$. PC, principal component.

Figure E4: **Cytokine levels in relation with time from symptoms onset and validation of the association between cytokine combinations and severity**. Kinetic evolution of IFN-α (**A**) and IL-6 (**B**) levels are plotted according to time from symptoms onset. Patients with very short or very long time from symptoms onset show a trend for higher or lower levels of these cytokines. **C**) PCA analysis performed with the same method as PCA in Figure 1B, but using only COVID-19 patients with symptoms onset between day 6 and 15. For these patients, there was no difference between the respiratory severity groups in the time from symptoms onset (as seen in Table 1), and no correlation of any cytokine levels with time from symptoms onset. Nevertheless, the same association between cytokine combinations and severity is observed as in Figure 1, thus validating the lack of effect of time from symptoms onset on this association. Ellipses represent the 68% confidence interval (CI) of the patient distribution in each group.

Figure E5: **Distinct cytokine profiles associated with COVID-19 respiratory severity in the validation cohort.** PCA of the 8 serum cytokines most contributing to inter-patient variation (as found for the initial cohort, Figure 1B) performed for the validation cohort patients (**A**) and for the validation and initial cohorts together (**B**) segregating the respiratory severity groups. Ellipses represent the 68% confidence interval of patient distribution in each group. Levels of the cytokine combination functions fcc-$_{INFLAM}$ (**C, D**) and fcc-$_{IFNI}$ (**E, F**), as defined from the analysis of the initial cohort, are depicted for each respiratory severity group for the validation cohort patients (**C, E**) and for the validation and initial cohorts together (**D, F**).

ns: non-significant; ✱ $p<0.05$; ✱✱ $p<0.01$; ✱✱✱ $p<0.001$; ✱✱✱✱ $p<0.0001$.

Figure E6: **Distinct cytokine combinations associated with COVID-19 mortality in the validation cohort.** PCAs using the cytokines identified as most contributing to the separation of surviving versus deceased patients in the initial cohort for each respiratory severity group:

No-MVS (**A**), MVS* (**B**) and ECMO (**C**), respectively, in both validation and initial cohorts together. Ellipses represent the 68% confidence interval of patient distribution in each group. Levels of the cytokine combination functions identified in the analysis of the initial cohort for each group: $f_{No\text{-}MVS}$ (**D**), f_{MVS} (**E**), and f_{ECMO} (**F**) are depicted for surviving versus deceased patients in each respiratory severity group in both validation and initial cohorts together.

MVS*: MVS with SAPS-II ≥ 35 (median of the MVS patients). Dec.: deceased.

ns: non-significant; ✱ p<0.05; ✱✱ p<0.01; ✱✱✱ p<0.001; ✱✱✱✱ p<0.0001.

Figure E7: **Receiver operating characteristic (ROC) curves of mortality outcome comparing all cytokine combination functions, SAPS and age**. ROC curves for $f_{No\text{-}MVS}$, f_{MVS}, f_{ECMO} and fcc_{INFLAM}, as well as SAPS and age are plotted for their predictive value of mortality in each of the respiratory severity groups: No-MVS (**A**), MVS with SAPS ≥35 (**B**) and ECMO (**C**) of the initial cohort. The ROC curve for fcc_{IFN} is equal to that of $f_{No\text{-}MVS}$.

Figure E8: **Receiver operating characteristic (ROC) curves of mortality outcome comparing the corresponding cytokine combination function per severity group with all individual cytokine levels.** ROC curves for $f_{No\text{-}MVS}$ in No-MVS patients (**A**), f_{MVS} in MVS (SAPS ≥35) patients (**B**) and f_{ECMO} in ECMO patients (**C**) of the initial cohort, are plotted together with the corresponding ROC curves for each cytokine individually and for SARS-CoV-2 antigen levels.