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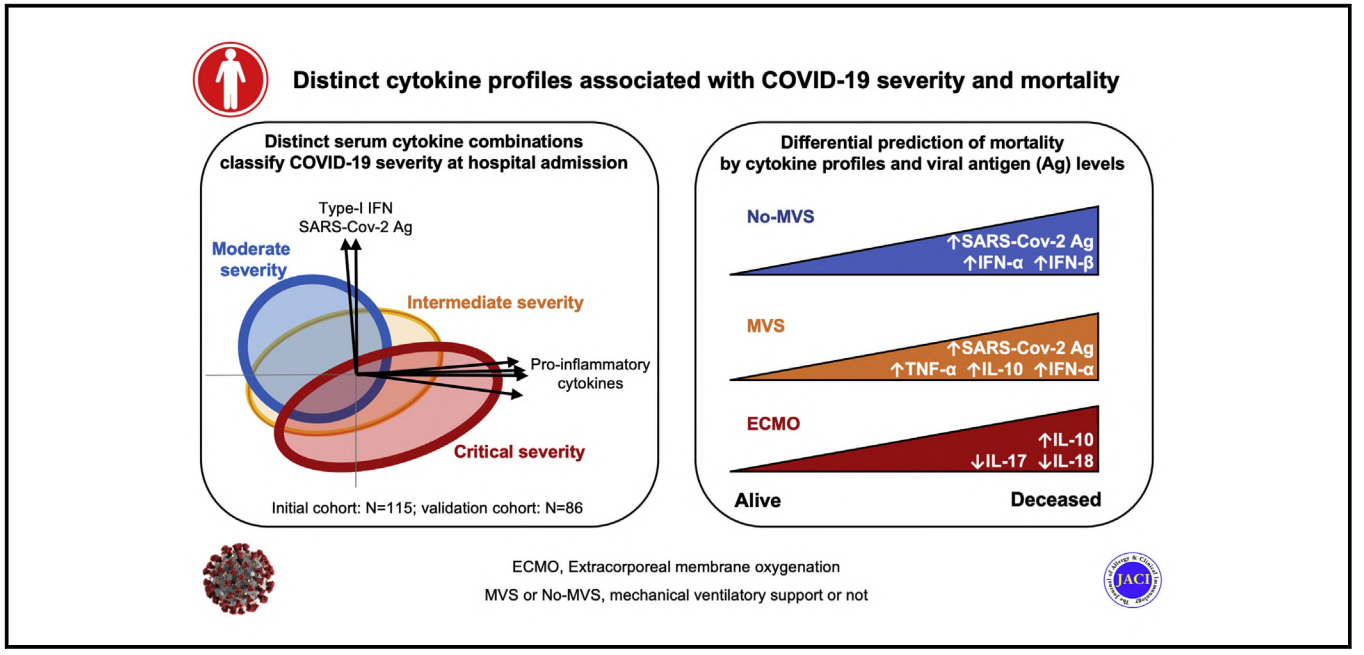
GRAPHICAL ABSTRACT



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Background: Markedly elevated levels of proinflammatory cytokines and defective type-I interferon responses were reported in patients with coronavirus disease 2019 (COVID-19).
Objective: We sought to determine whether particular cytokine profiles are associated with COVID-19 severity and mortality.
Methods: Cytokine concentrations and severe acute respiratory syndrome coronavirus 2 antigen were measured at hospital admission in serum of symptomatic patients with COVID-19 (N = 115), classified at hospitalization into 3 respiratory severity groups: no need for mechanical ventilatory support (No-MVS), intermediate severity requiring mechanical ventilatory support (MVS), and critical severity requiring extracorporeal membrane oxygenation (ECMO). Principal-component analysis was used to characterize cytokine profiles associated with severity and mortality. The results were thereafter confirmed in an independent validation cohort (N = 86).
Results: At time of hospitalization, ECMO patients presented a dominant proinflammatory response with elevated levels of TNF-α, IL-6, IL-8, and IL-10. In contrast, an elevated type-I interferon response involving IFN-α and IFN-β was characteristic of No-MVS patients, whereas MVS patients exhibited both profiles. Mortality at 1 month was associated with higher levels of proinflammatory cytokines in ECMO patients, higher levels of type-I interferons in No-MVS patients, and their combination in MVS patients, resulting in a combined mortality prediction accuracy of 88.5% (risk ratio, 24.3; $P < .0001$). Severe acute respiratory syndrome coronavirus 2 antigen levels correlated with type-I interferon levels and were associated with mortality, but not with proinflammatory response or severity.
Conclusions: Distinct cytokine profiles are observed in association with COVID-19 severity and are differentially predictive of mortality according to oxygen support modalities. These results warrant personalized treatment of COVID-19 patients based on cytokine profiling.

Key words: *COVID-19, serum cytokines, type-I interferons, respiratory severity, mortality, principal-component analysis*

Coronavirus disease 2019 (COVID-19) is a recently emerged global infectious respiratory disease that is caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), a novel betacoronavirus.[1,2] Interindividual clinical variability over the course of SARS-CoV-2 infection is notorious, and prognosis remains difficult to predict at hospital entry. Respiratory failure is the most prominent feature associated with severe COVID-19, but severe damage to other organs is also observed.[1,3] Many treatments have been tried to date, albeit with mixed results, which might be linked with the clinical and biological heterogeneity of the disease.

COVID-19 has been associated with the onset of a "cytokine storm",[4] an abnormal regulation and exuberant release of several proinflammatory cytokines at inappropriate time intervals during infection.[5] It remains however unclear whether all patients present with the same type of cytokine release syndrome. We therefore postulated that focusing on separate cytokine levels might give only a partial view of a complex and interlinked inflammatory response that might not account for the various clinical profiles associated with COVID-19.

Abbreviations used
COVID-19: Coronavirus disease 2019
f_{CC}: Cytokine combination function
ECMO: Extracorporeal membrane oxygenation
MVS: Mechanical ventilatory support
PCA: Principal-component analysis
SAPS-II: Simplified Acute Physiology Score
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

In the present study, we used highly sensitive, classical or digital, multiplex ELISA technologies to analyze combined cytokine production profiles in serum of patients with COVID-19 at the time of hospital admission. Thereafter, we used a nonsupervised bioinformatics approach to identify the cytokine combinations that represent a reliable biomarker of COVID-19–related pulmonary severity and/or mortality.

METHODS

Patients and samples

This prospective study was performed at Pitié-Salpêtrière Hospital in Paris and approved by the local ethical committee, *Comité d'Ethique de la Recherche* (CER-SU), of Sorbonne University (#CER-2020-21 and -31). Patients with real-time RT-PCR–confirmed SARS-CoV-2 RNA presence in their nasopharyngeal swab, and pulmonary manifestations, were included after admission to intensive care units or to the Internal Medicine department.

A total of 115 patients with COVID-19 (demographic and clinical characteristics in Table I and Table E1 in this article's Online Repository at www.jacionline.org) were enrolled between March 17 and May 11, 2020, in the initial cohort. Patients were divided at day of hospitalization into 3 groups according to severity of pulmonary involvement: (1) moderate severity treated with no oxygen support, nasal cannula, or oxygen mask (No-MVS, N = 34), (2) intermediate severity requiring mechanical ventilatory support (MVS, N = 50), and (3) critical respiratory failure requiring extracorporeal membrane oxygenation (ECMO, N = 31). At 1 month after admission, 7 (20.6%), 19 (38%), and 7 (22.6%) patients were deceased in the No-MVS, MVS, and ECMO groups, respectively. In addition, healthy SARS-CoV-2–negative individuals (N = 10) were included as controls.

A validation cohort included 86 patients with COVID-19 (clinical characteristics in Table E2 in this article's Online Repository at www.jacionline.org) enrolled between June 28 and November 23, 2020. Patients were classified into respiratory severity groups as in the initial cohort (No-MVS: N = 10, MVS: N = 58, and ECMO: N = 18). New therapeutic guidelines[6] led to a decrease in mortality: No-MVS: 0 (0%), MVS: 16 (27.6%), and ECMO: 4 (22.2%). In particular, all patients in the validation cohort received glucocorticoid treatment.

Serum samples in the initial cohort were all collected at the day of hospital admission. However, in the validation cohort, samples were collected over the first 2 weeks after hospitalization (median, 5 days). Only patients with sample collected at most 21 days after symptom onset were included in the analysis (median, 9 and 12 days in initial and validation cohorts, respectively).

Immunoassays

Serum cytokine concentrations were determined using the Quanterix platforms (IFN-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17A, IL-18, IL-22, GM-CSF, and TNF-α) and ELISA (IFN-β) from PBL Assay Science.

Serum nucleocapsid (N) antigen concentrations were determined using the COV-QUANTO ELISA kit (AAZ, Boulogne Billancourt, France).

Procedures and specificity of the immunoassays used in this work are detailed in this article's Methods section in the Online Repository at www.jacionline.org.

TABLE I. Demographic characteristics, baseline characteristics, and clinical outcomes of 115 patients with COVID-19 in the initial cohort

Characteristic	No-MVS (N = 34)	MVS (N = 50)	ECMO support (N = 31)	All patients (N = 115)	*P* value*
Age (y), median (IQR)	73 (46-73)	63 (55-69)	49.5 (42-56)	58 (49-66)	**<.001**
Sex: male, n (%)	22 (64.7)	36 (72)	25 (83.3)	83 (72.2)	.36
Severity score at baseline					
SAPS-II, median (IQR)	26 (18-33)	35 (27-44)	52 (45-65)	36 (26-49)	**<.001**
SOFA, median (IQR)	—	7 (4-7)	12 (9-15)	—	**<.001**
Respiratory severity					
Nasal cannula or high-concentration mask	25 (73.5)	—	—	25 (21.7)	
Noninvasive ventilation or high-flow nasal cannula	—	10 (20)	—	10 (8.7)	
Invasive mechanical ventilation	—	40 (80)	31 (100)	71 (61.7)	
ECMO	—	—	31 (100)	31 (27)	
Time from onset of symptoms to admission/sample					
Median days, n (IQR)	8 (2-11)	9 (7-11)	12 (8-15)	9 (6-12)	**.001**
Past medical history, n (%)					
Cardiovascular disease	11 (32.4)	10 (20)	2 (6.5)	23 (20)	**.03**
Type 2 diabetes	11 (32.4)	19 (38)	12 (38.7)	42 (36.5)	.83
Obesity (≥30)	7 (20.6)	18 (36)	19 (61.3)	44 (38.3)	**.03**
Hypertension	19 (55.9)	29 (58)	17 (54.8)	65 (56.5)	.96
Any condition†	28 (82.4)	41 (82)	28 (90.3)	97 (84.3)	.57
Clinical outcome at 1 mo (%)					
Discharged	27 (79.4)	30 (60)	20 (64.5)	77 (67)	.17
Remained in hospital	0 (0)	1 (2)	4 (12.9)	5 (4.3)	.25
Deceased	7 (20.6)	19 (38)	7 (22.6)‡	33 (28.7)‡	.23
Length of stay§ (d), median (IQR)	10 (6-16)	10 (7-22)	32 (25-55)	13 (7-29)	**<.001**

SOFA, Sequential Organ Failure Assessment.
Boldface values indicate statistical significant differences between the groups.
*Kruskal-Wallis test for continuous variables; Fisher exact test for discrete variables.
†Including hypertension, diabetes, obesity, cancer, arterial thromboembolism, venous thromboembolism, systemic autoimmune disease, HIV infection, solid organ transplant, chronic pulmonary disease, chronic liver disease, chronic kidney disease, chronic neurologic disease, and cardiovascular disease.
‡Two additional patients died later than day 30 of hospitalization (at day 46 and day 50).
§As of June 18, 2020.

Statistical and bioinformatics analysis

Principal-component analysis (PCA) was used to determine the cytokine combinations associated with severity and mortality. Statistical significance of differences between groups was assessed using the nonparametric Mann-Whitney and Kruskal-Wallis tests for continuous variables and Fisher exact test for frequencies. Correlations were assessed by the nonparametric Spearman test. Analyses were performed with SPSS, GraphPad-Prism, and R. Two-sided *P* value less than .05 was considered statistically significant (ns: nonsignificant; *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$).

For detailed methods, please see this article's Methods section in the Online Repository.

RESULTS

Patients' severity and mortality

Patients with COVID-19 in the initial cohort (N = 115) were classified into 3 respiratory severity groups: no need for mechanical ventilatory support (No-MVS), intermediate severity requiring MVS, and critical severity requiring extracorporeal membrane oxygenation (ECMO). Mortality at 1 month after admission (28.7%) was not different between the 3 severity groups ($P = .23$). The Simplified Acute Physiology Score (SAPS-II: median, 36; interquartile range, 26-49), which classifies disease severity for patients admitted to intensive care units,[7] validated our respiratory severity classification, with median SAPS-II of 26, 35, and 52 for No-MVS, MVS, and ECMO groups, respectively ($P < .001$) (see Fig E1 in this article's Online Repository at www.jacionline.org). Patients' SAPS-II scores were associated with mortality only in the MVS group (Fig E1).

Distinct cytokine profiles associated with COVID-19 severity

Concentrations of 12 cytokines were measured in serum from the patients with COVID-19 at hospital admission, and in healthy SARS-CoV-2–negative subjects (see Fig E2 in this article's Online Repository at www.jacionline.org). As expected, PCA emphasized important overall cytokine profile differences ($P < .001$) between patients with COVID-19 and healthy controls, but also underlined the heterogeneity of the COVID-19 group (Fig 1, *A*). **[F1-4/C]**

To elucidate the association of serum cytokine levels with COVID-19 severity, we performed nonsupervised PCA among patients with COVID-19 only, highlighting 2 distinct cytokine combinations (Fig 1, *B*). Proinflammatory cytokines, including TNF-α, IL-6, IL-8, and IL-1β, as well as IL-10 and IL-22, mainly contributed to the first principal component, whereas the type-I interferon antiviral cytokines IFN-α and IFN-β had an orthogonal contribution on the second principal component. Strikingly, these 2 distinct cytokine combinations were differentially associated with COVID-19 respiratory severity, with 96.4% of ECMO patients and 96.6% of No-MVS patients appropriately segregated by the separatrix line ($P < .0001$).

Taking into account only the cytokines with the greatest contribution to the principal components (Fig 1, *B*), 2 cytokine combination functions (f_{CC}) were constructed: $fcc\text{-}_{INFLAM}$, based on the proinflammatory cytokines TNF-α, IL-6, IL-8, and IL-10, and $fcc\text{-}_{IFNI}$, based on the type-I interferons IFN-α and IFN-β (see further information in this article's Online Repository at www.jacionline.org). These 2 cytokine combinations were distinctively

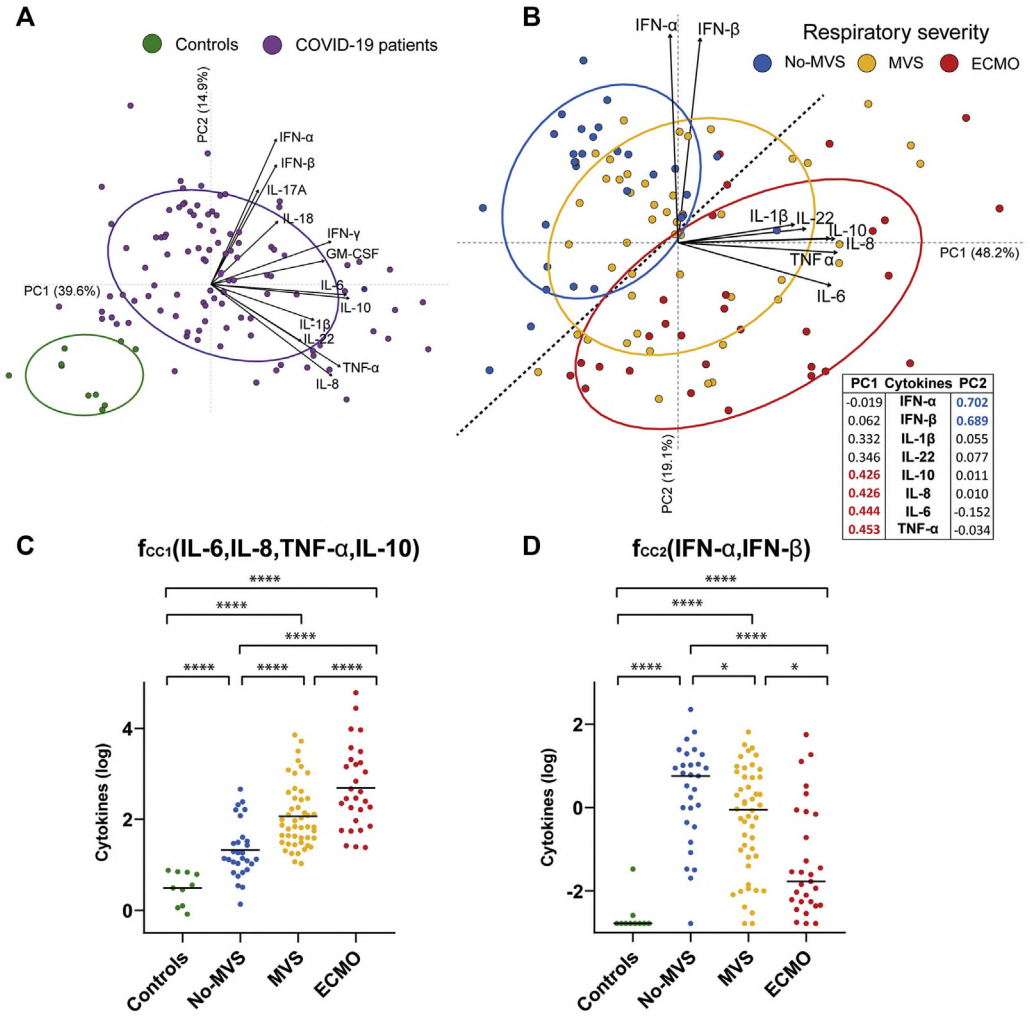

FIG 1. Distinct cytokine profiles associated with COVID-19 respiratory severity. **A**, PCA of 12 serum cytokines measured in patients with COVID-19 and controls. **B**, PCA, and PC factors (table), of the 8 cytokines most contributing to interpatient variation in patients with COVID-19, segregates the patients by respiratory severity groups. Ellipses represent the 68% CI of patient distribution in each group. Levels of the ensuing fcc-$_{INFLAM}$ (**C**), for inflammatory cytokines derived from PC1, and fcc-$_{IFNI}$ (**D**), for type-I interferons derived from PC2, are depicted for each respiratory severity group and the controls. Initial PCA with all 12 cytokines measured in the patients with COVID-19 had shown that IFN-γ, GM-CSF, IL-17A, and IL-18 either contribute less to the variation between patients and/or have a mixed contribution to PC1 and PC2. PC1 and PC2 contributed most of the variation between patients (67.3%), whereas higher PC dimensions had lower contributions (PC3 = 9.7%, PC4 = 6.6%) and did not contribute to separation of patients by severity. *ns*, Nonsignificant; *PC*, principal component. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

associated with severity: fcc-$_{INFLAM}$ values were higher in ECMO patients ($P < .0001$: Fig 1, *C*), whereas fcc-$_{IFNI}$ values were higher in No-MVS patients ($P < .0001$; Fig 1, *D*), in a mutually exclusive manner.

The cytokine profiles of the MVS patients were scattered in between those of the 2 other groups (Fig 1, *B*), showing intermediate levels of both fcc-$_{INFLAM}$ and fcc-$_{IFNI}$ (Fig 1, *C* and *D*). Indeed, analysis by k-means clustering revealed that MVS patients could be divided into 3 subgroups ($P < .01$) with different combinations of elevated fcc-$_{INFLAM}$ and/or fcc-$_{IFNI}$ values (see Fig E3 in this article's Online Repository at www.jacionline.org).

Repeating the same analysis with severity groups defined by SAPS-II scores showed the same association of fcc-$_{INFLAM}$ and fcc-$_{IFNI}$ with COVID-19 severity (Fig E1, *B*). Moreover, these results were confirmed for patients with intermediate time lapse from symptom onset (see Fig E4 in this article's Online Repository at www.jacionline.org), for whom there was no difference in this time lapse between the groups (Table I) and no variation in cytokine levels as function of time from symptom onset (Fig E4). Logistic regression analysis taking into account all baseline factors (Table E1) confirmed that fcc-$_{INFLAM}$ and fcc-$_{IFNI}$ were the only factors remaining associated with severity in a statistically significant manner after multifactorial correction.

Furthermore, our results were confirmed in an independent validation cohort (N = 86). PCA of the validation cohort patients alone (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org), or both cohorts combined (Fig E5, *B*), showed again segregation of ECMO patients having higher levels of

proinflammatory cytokines and No-MVS patients having higher levels of type-I interferon response. Accordingly, the values of fcc-$_{INFLAM}$ and fcc-$_{IFNI}$ were mutually exclusive, and significantly different, between the respiratory severity groups for the validation cohort alone (Fig E5, *C* and *E*) or for both cohorts together (Fig E5, *D* and *F*).

COVID-19 mortality risk is associated with distinct cytokine profiles

To elucidate the association of serum cytokine levels at time of hospitalization with COVID-19 mortality 1 month later, we performed PCA for each oxygen support modality separately, because PCA for all patients together did not show separation between alive and deceased patients. Mortality was differentially associated with distinct cytokine combinations in each group. In ECMO patients, mortality was associated with elevated levels of proinflammatory cytokines (in particular IL-10, IL-8, IL-6, and TNF-α), as well as with decreased levels of IL-17A and IL-18 (Fig 2, *C*). However, in No-MVS patients, elevated levels of IFN-α and IFN-β, but not proinflammatory cytokines, were associated with mortality (Fig 2, *A*). Lastly, in MVS patients with SAPS-II score greater than or equal to 35 (median of the MVS group, below which only 4% of MVS patients deceased), mortality was associated with the combination of elevated levels of both proinflammatory cytokines (in particular TNF-α and IL-10) and IFN-α (Fig 2, *B*).

By selecting the cytokines most associated with mortality according to the principal-component factors (Fig 2, *A-C*), we defined an f_{CC} for each group: $f_{No\text{-}MVS}$ based on IFN-α and IFN-β for No-MVS patients (Fig 2, *D*), f_{MVS} based on TNF-α, IL-10, and IFN-α for MVS patients with SAPS-II score greater than or equal to 35 (Fig 2, *E*), and f_{ECMO}, based on IL-10, IL-17A, and IL-18 for ECMO patients (Fig 2, *F*). The magnitude of each of these cytokine combinations was significantly higher in deceased as compared with alive patients in each corresponding severity group, but not in the other groups. Furthermore, regression analysis with other cofactors such as demographic characteristics, past medical history, and COVID-19–related treatment (Table E1) showed that $f_{No\text{-}MVS}$, f_{MVS}, and f_{ECMO} were the only factors that remained significantly associated with mortality after multifactorial correction per each group accordingly.

The differential association of mortality with the different f_{CC} identified in the initial cohort was further confirmed in an independent validation cohort (N = 86). PCA for each of the severity groups (No-MVS, MVS, and ECMO), using only the cytokines in each f_{CC} ($f_{No\text{-}MVS}$, f_{MVS}, and f_{ECMO}) accordingly, separated deceased and alive patients for the initial and validation cohorts together (see Fig E6, *A-C*, in this article's Online Repository at www.jacionline.org). Furthermore, values of $f_{No\text{-}MVS}$, f_{MVS}, and f_{ECMO} from both cohorts together showed a significant difference between alive and deceased patients, but only for the f_{CC} of the corresponding group (Fig E6, *D-F*).

Next, we set appropriate thresholds (selected to optimize accuracy of prediction, Fig 2) for each of the f_{CC} ($f_{No\text{-}MVS}$, f_{MVS}, and f_{ECMO}) and generated the prediction table and scores for each group accordingly (Table II). The overall mortality of patients with COVID-19 at 1 month after admission could be predicted by measuring circulating levels of only 6 cytokines, as well as the SAPS-II score for the MVS group, with an accuracy of 88.5%, a sensitivity of 93.3%, and a specificity of 86.5% (risk ratio, 24.3; odds ratio, 89.6; $P < .0001$; Table II).

To investigate the robustness of the predictions, we plotted receiver-operating characteristic curves of mortality outcome comparing the 3 f_{CC}, SAPS-II score, and age (see Fig E7 in this article's Online Repository at www.jacionline.org), as well as all individual cytokine levels (see Fig E8 in this article's Online Repository at www.jacionline.org) in each of the respiratory severity groups. In each group, the corresponding prediction function always represented the largest area under the curve. Moreover, to evaluate the robustness of the differential predictions, we calculated the relative sensitivity (rSens) and relative specificity (rSpec) of the corresponding function for each group and compared them with the 2 other functions (see Table E4 in this article's Online Repository at www.jacionline.org). The combined relative predictive score (rSens × rSpec) was always larger than 1.6, indicating that each function indeed best predicted mortality for the corresponding group.

High viral load associated with mortality in No-MVS and MVS, but not in ECMO, patients

SARS-CoV-2 nucleocapsid (N)-antigen levels were measured by ELISA[8] in 179 patients on the same day as the cytokine measurement. However, in 32 patients with sampling later than 14 days after symptom onset, antigen levels were significantly lower ($P < .001$). Thus, for the measurement of SARS-CoV-2 antigen, only the 147 patients sampled up to 14 days after symptom onset were considered.

Viral load was not associated with COVID-19 severity, neither as defined by oxygen support modality groups, nor by SAPS-II scores (Fig 3). However, mortality 1 month after hospitalization was associated with higher levels of antigen (Fig 3, *A*) in No-MVS patients ($P = .002$) and MVS patients ($P = .008$), but not in ECMO patients.

Viral load is correlated with type-I interferon response but not with inflammatory cytokines

SARS-CoV-2 antigen levels were significantly correlated with the type-I interferon cytokine combination fcc-$_{IFNI}$ (Fig 4) in No-MVS patients ($R = 0.79$; $P < .001$) and in ECMO patients ($R = 0.57$; $P = .002$), as well as with IFN-α and IFN-β individually, but not with IFN-γ. However, in intermediate severity MVS patients, antigen levels were not correlated with those of IFN-α and IFN-β individually, nor with the fcc-$_{IFNI}$ cytokine combination, but were correlated with IFN-γ levels instead ($R = 0.45$; $P < .001$). However, inflammatory cytokine levels, either individually or in the cytokine combination fcc-$_{INFLAM}$, were not significantly correlated with viral load. Interestingly, deceased No-MVS and MVS patients (Fig 4, *A* and *B*), but not ECMO patients (Fig 4, *C*), had both higher levels of antigen and type-I interferons.

Association of cytokine levels and SARS-CoV-2 viral load with baseline conditions

Major baseline demographic parameters associated with COVID-19 severity or mortality[1] (sex, age, smoking, body mass index, obesity, and overweight) were neither significantly associated with SARS-CoV-2 viral load nor with any of the cytokine levels or their combinations. Also, most baseline clinical conditions (cancer,

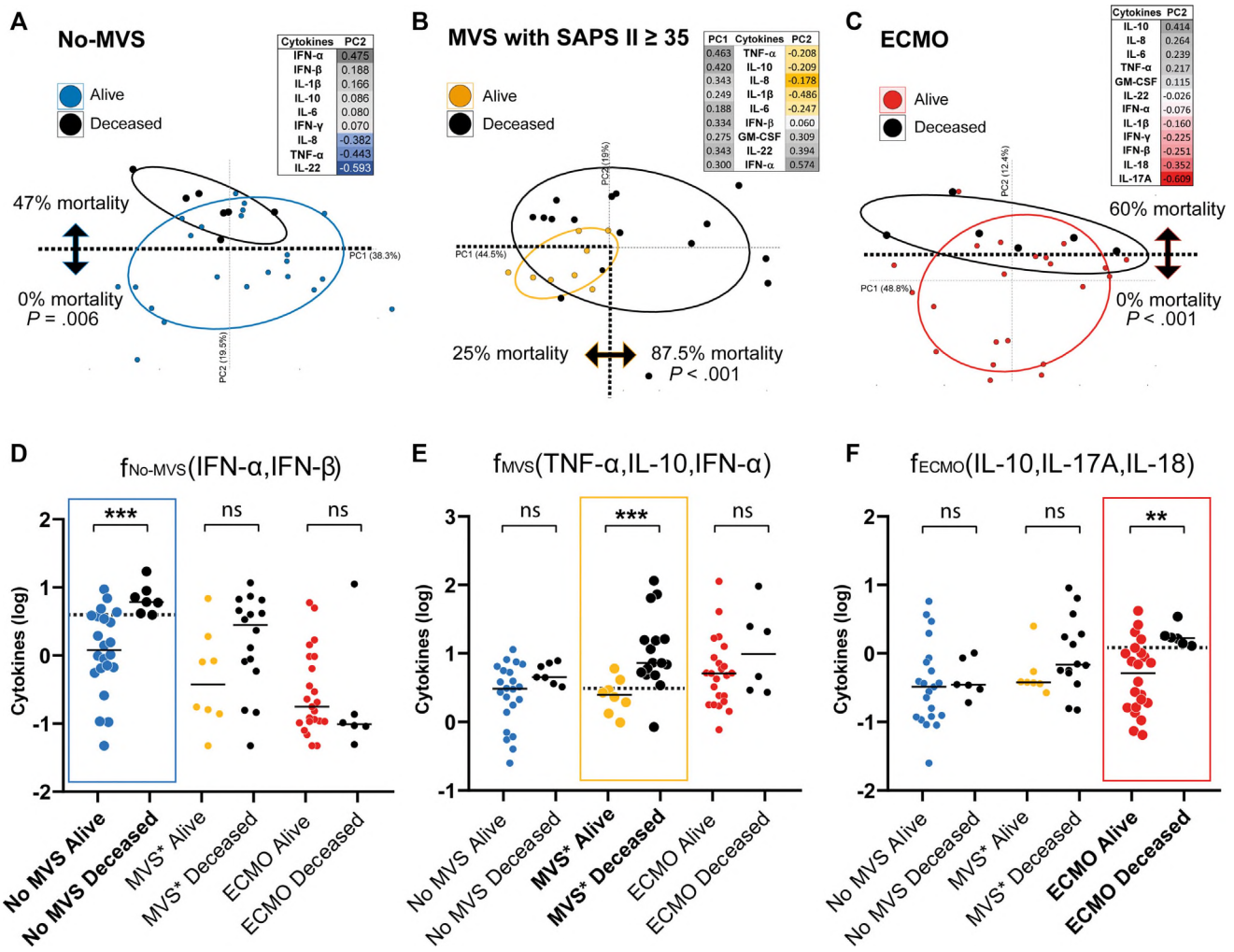

FIG 2. Distinct cytokine combinations associated with COVID-19 mortality. PCAs for each respiratory severity group: No-MVS (**A**), MVS(*) (**B**), and ECMO (**C**) segregate surviving vs deceased patients in association with different cytokine combinations (corresponding PC factors in the tables). Levels of the ensuing f_{CC} from the PC factors for each group, $f_{No\text{-}MVS}$ (**D**), f_{MVS} (**E**), and f_{ECMO} (**F**), are depicted for surviving vs deceased patients in each respiratory severity group. *ns*, Nonsignificant; *PC*, principal component. (*)MVS with SAPS-II score greater than or equal to 35 (median of the MVS patients). *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

immunodeficiency, chronic pulmonary disease, asthma, chronic obstructive pulmonary disease, chronic liver disease, chronic neurologic disease) did not show a significant association with SARS-CoV-2 antigen levels or with any of the cytokine levels or their combinations. However, we found that chronic kidney disease was significantly ($P = .002$) associated with higher SARS-CoV-2 antigen and IFN-α ($P < .001$) levels. Patients with diabetes, cardiovascular disease, and hypertension also showed a trend for higher viral load and IFN-α levels, but this was not significant when considering the occurrence of kidney disease.

Altogether, the lack of association between major baseline demographic parameters and any of the cytokine levels or their combinations further emphasizes the interest of cytokine profiling for COVID-19 patient stratification and monitoring.

DISCUSSION

Our finding of distinct cytokine profiles and their correlation with SARS-CoV-2 viral load sheds light on factors associated with COVID-19 severity and mortality. Critically severe patients (requiring ECMO and/or with the highest SAPS-II scores) do not have higher viral load than less severe patients, but rather exhibit higher levels of inflammatory cytokines ($fcc\text{-}_{INFLAM}$) and lower type-I interferon response ($fcc\text{-}_{IFNI}$). Accordingly, mortality in ECMO patients is not associated with higher viral loads or higher type-I interferon levels, but rather with the combination of high IL-10 (as well as TNF-α, IL-6, and IL-8) levels and low IL-17A and IL-18 levels (f_{ECMO}).

However, moderate severity patients requiring no MVS (or patients with the lowest SAPS-II scores at admission) exhibit lower inflammatory cytokine levels but a higher type-I interferon response. Mortality in this No-MVS group is strongly associated with higher viral loads and high type-I interferon levels, with the latter strongly correlated with antigen levels. It is unclear which of the 2 parameters constitutes the most important risk factor in this group, but, interestingly, even among patients with high viral load, mortality was still found to be associated with exacerbated type-I interferon response ($f_{No\text{-}MVS}$).

TABLE II. Prediction of COVID-19 mortality at 1 month from admission using cytokine combinations measured at hospital admission

	Mortality prediction for different groups*			
	No-MVS	**MVS**	**ECMO**	**All**
Predictor	$f_{NO\text{-}MVS}$(IFN-α, IFN-β)	f_{MVS}(SAPS, TNF-α, IL-10, IFN-α)	f_{ECMO}(IL-10, IL-17A, IL-18)	$f_{NO\text{-}MVS}$, f_{MVS}, f_{ECMO}
N†	29	47	28	104
Mortality	24.1%	36.2%	21.4%	28.9%
Accuracy	86.2%	91.5%	85.7%	88.5%
NPV	100%	93.3%	100%	97.0%
PPV	63.6%	88.2%	60.0%	73.7%
Specificity	81.8%	93.3%	81.8%	86.5%
Sensitivity	100%	88.2%	100%	93.3%
OR [95% CI]	> 29.8 [2.8-315.6]	105 [13.4-822.1]	> 25.5 [2.4-275.7]	89.6 [18.4-435.8]
RR [95% CI]	> 11.5 [1.6-81.0]	13.2 [3.4-51.1]	> 10.8 [1.5-77.5]	24.3 [6.1-96.5]
P value‡	.0002	<.0001	.0006	<.0001

NPV, Negative predictive value; *OR*, odds ratio; *PC*, principal component; *PPV*, positive predictive value; *RR*, risk ratio.

*Prediction of mortality was performed using following functions:

No-MVS: $f_{No\text{-}MVS} = (0.475 \times \text{Log(IFN-}\alpha) + 0.188 \times \text{Log(IFN-}\beta)) > 0.59$

MVS: SAPS-II score ≥ 35 & $f_{MVS} = (0.474 \times \text{Log(TNF-}\alpha) + 0.444 \times \text{Log(IL-10)} + 0.194 \times \text{Log(IFN-}\alpha)) > 0.5$

where SAPS-II score =35 is the median for MVS patients.

ECMO: $f_{ECMO} = (0.414 \times \text{Log(IL-10)} - 0.609 \times \text{Log(IL-17A)} - 0.352 \times \text{Log(IL-18)}) > 0.1$

All: Each patient was predicted by the predictor for the corresponding oxygen support received.

The coefficients are taken from corresponding PC factors in Fig 2.

†Some of the numbers are lower than those in Table I because of missing cytokine data in few patients.

‡Statistical significance of the prediction was assessed by the Fisher exact test.

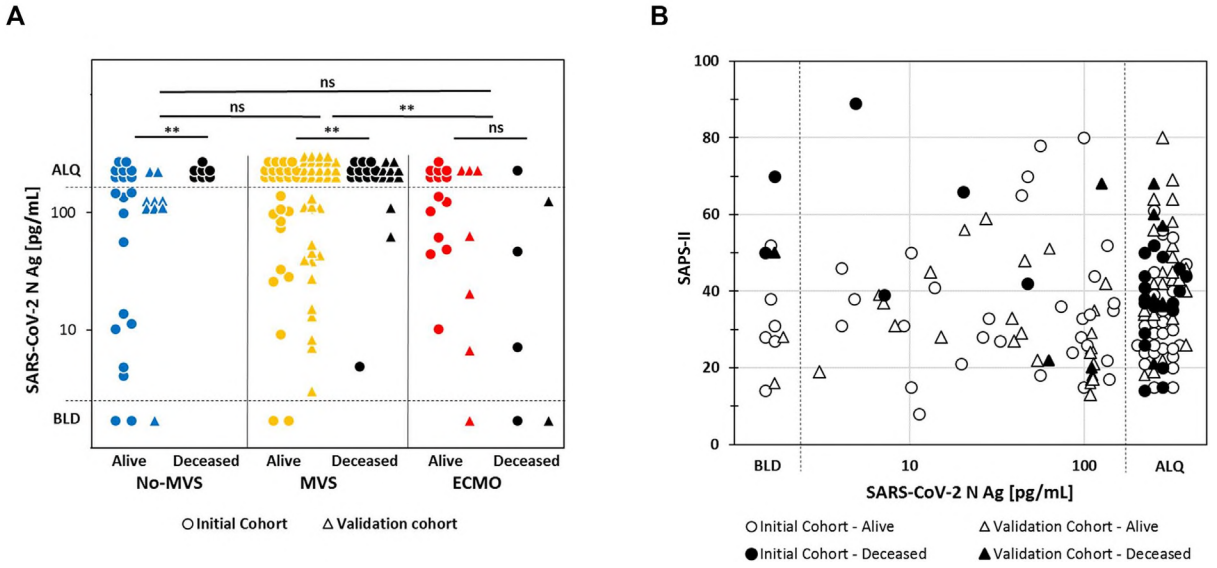

FIG 3. SARS-CoV-2 serum viral load association with severity and mortality. **A,** SARS-CoV-2 nucleocapsid (N)-antigen levels depicted by respiratory severity group and deceased vs surviving patient per group (No-MVS: 100% ALQ in deceased vs 34.5% ALQ in surviving patients, $P = .002$; MVS: 86.4% ALQ in deceased vs 53.2% ALQ in surviving patients, $P = .008$; ECMO: 16.7% ALQ in deceased vs 47.6% ALQ in surviving patients, P = ns). **B,** SAPS-II scores depicted as function of N-antigen levels per patient ($R = 0.05$; P = ns), with deceased vs surviving patients marked. Only patients with a sample taken at most 14 days after symptom onset are included in the analysis, because antigen levels were significantly lower in samples taken after 14 days (50% BLD), as compared with until day 14 (5.4% BLD; $P < .0001$). For samples until 14 days there was no correlation of N-antigen levels with time from symptom onset and no difference between the cohorts. *Ag*, Antigen; *ALQ*, above upper limit of quantification; *BLD*, below limit of detection; *ns*, nonsignificant. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

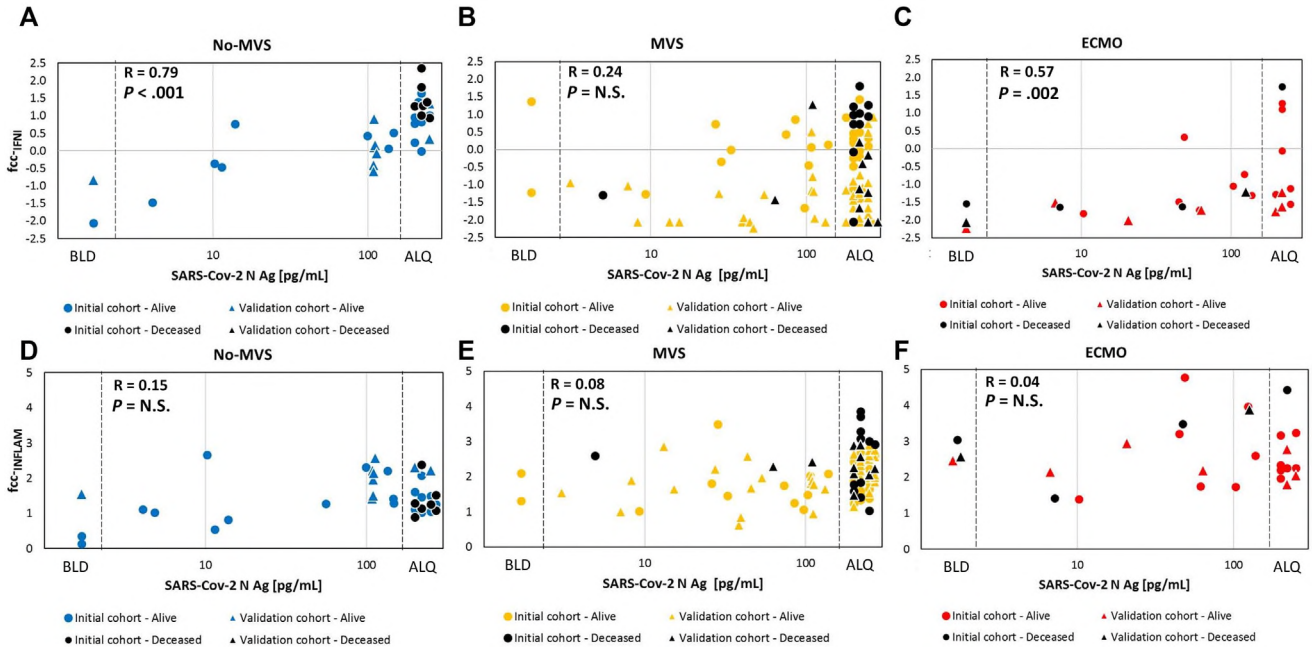

FIG 4. Correlation between SARS-CoV-2 serum viral load and cytokine levels. The f_{CC} values for fcc-$_{IFNI}$ comprising type-I interferons IFN-α, and IFN-β (**A-C**) and fcc-$_{INFLAM}$ comprising the inflammatory cytokines TNF-α, IL-10, IL-6, and IL-8 (**D-F**) are plotted as function of SARS-CoV-2 nucleocapsid (N)-antigen levels for each of the respiratory severity groups. Only patients with a sample taken at most 14 days after symptom onset are included in the analysis (see Fig 3). *Ag*, Antigen; *ALQ*, above upper limit of quantification; *BLD*, below limit of detection; *N.S.*, nonsignificant.

Lastly, intermediate severity patients (MVS or intermediate SAPS-II scores) seem to comprise a mixed population with a large, intermediate span of both fcc-$_{INFLAM}$ and fcc-$_{IFNI}$ levels. Mortality in MVS patients is associated with higher levels of both inflammatory cytokines and type-I interferons (f_{MVS}) as well as higher viral loads. Indeed, we show that MVS patients can be clustered into at least 3 different subgroups in terms of their cytokine profiles (Fig E3). Thus, instead of considering the cytokine profile of each group, it may be more correct to classify severity and assess mortality risk on the basis of individual levels of the cytokine combinations identified here, together with viral load.

The association of enhanced serum cytokine levels with mortality does not necessarily imply cytokine-related toxicity. IL-6, IL-8, and TNF-α are known to have deleterious proinflammatory effects, whereas IL-10 is considered to mainly dampen inflammation,[9] although its expression levels were found to be associated with higher mortality rates in patients requiring MVS and ECMO. These results do not point to a causal effect but to an association of the cytokine profiles with mortality. Elevated levels of other circulating cytokines were also associated with a favorable outcome of the disease, such as high IL-17A and IL-18 levels in ECMO patients (Fig 2, *C*, and Fig E8), possibly in relation with their protective role in antimicrobial responses.[10,11]

It is worth mentioning that there are no clear differences in the cause of death between the different severity groups. Nevertheless, in the no-MVS and MVS groups, 32% and 20%, respectively, of the patients had a history of cardiovascular disease (Table E1), and mortality was strongly associated with high levels of type-I IFNs, which reportedly have toxic effects on the cardiovascular system.[12,13] Conversely, among MVS and ECMO patients, 30% and 32% were overweight, and 36% and 61% were obese, respectively (Table E1). Obesity was strongly associated with a chronic inflammatory state predisposing to an exacerbated cytokine response following viral infection that may drive the development of septic shock, acute respiratory distress syndrome, pulmonary embolism, and multiorgan failure.[14]

The results from the present study corroborate previous reports showing that some critically severe patients with COVID-19 are characterized by defective type-I IFN responses.[5,15,16] Indeed, we recently contributed to report patients with undetectable systemic concentrations of IFN-α, either in the context of genetic inborn errors of cytokine-mediated immunity[17] or dependent on the presence of anticytokine autoantibodies.[18] Nevertheless, IFN-α was detectable at high levels within the first 7 days after the onset of symptoms also in severe patients (Figs E2 and E4). Similarly, although levels of IFN-β were low in many patients with COVID-19, possibly due to a lower sensitivity of detection (see Table E3 in this article's Online Repository at www.jacionline.org), they were nevertheless not systematically undetectable in severe patients (Fig E2), contrary to what has been previously suggested.[16,19] We show that mortality in No-MVS patients is rather associated with exacerbated type-I IFN responses. Although it is possible that elevated serum interferon levels would result from downstream signaling defects in the type-I IFN pathway,[20] previous results suggest that an actively persisting type-I IFN response is associated with immunopathology.[21,22] Of note, an elevated type-I interferon response in these patients was highly correlated with elevated viral load (Fig 4), and thus could simply reflect an appropriate response to viral replication. Although we did not find evidence that an exacerbated type-I interferon response was related to cardiologic (or other) specific complications in No-MVS and MVS patients (Tables E1 and E2), it should be emphasized that mortality in these groups was associated with the highest type-I interferon levels.

Therapeutic approaches in patients with COVID-19 targeting only 1 type of cytokine, such as IL-6 or GM-CSF, have been reported not to be effective for preventing death.[23,24] Moreover, targeting IL-1β could even be harmful, because an excess of premature deaths was recently reported in patients with COVID-19 receiving an IL-1 receptor antagonist.[25] Indeed, we found no association between GM-CSF or IL-1β and mortality. Furthermore, IL-6 was only mildly associated with mortality in ECMO patients, as was TNF-α, arguing that the use of antagonists targeting only these cytokines might not be best suited for all patients with life-threatening COVID-19, contrasting with a recent suggestion.[26]

Our findings could also explain why the administration of glucocorticoids, in particular dexamethasone, which decreases blood levels of many cytokines, was successful in both patients receiving invasive mechanical ventilation (MVS) and those receiving oxygen without invasive mechanical ventilation, corresponding to part of No-MVS patients.[6,27] Because mortality in these patients was found to be strongly associated with high levels of type-I IFNs, the modulation of secretion and/or activity of the latter cytokines could be related to corticosteroid efficacy.[28]

The present study shows that cytokine profiling may have clinical implications for improved personalized treatment. For example, no-MVS patients do not present with enhanced levels of circulating inflammatory cytokines, such as IL-6 or TNF-α, thereby arguing that treatment with antagonists of the latter cytokines is not suitable for this group of patients. Moreover, profiling type-I IFN and anti-IFN antibodies in the serum of patients with COVID-19 may contribute to the selection of those who are most likely to benefit from IFN-β therapy.[29]

Associations of cytokine combinations with COVID-19 severity and mortality were confirmed in an independent, second pandemic wave validation cohort. It should be noted that these patients had all received corticosteroids at the time of sampling (in contrast to the patients of the initial cohort). The observed associations hold both with and without corticosteroid treatment. The mortality prediction table presented here was calculated only on the basis of initial cohort results, because those were measured at day of hospitalization, unlike the validation cohort. Receiver-operating characteristic curve analysis and the 95% CI of the odds ratios show the robustness of the mortality predictions per severity group. Altogether, on the basis of 6 cytokines measured at day of hospitalization, we obtained a highly accurate (88.5%) mortality prediction with 93.3% sensitivity and 86.5% specificity (positive predictive value = 73.7% and negative predictive value = 97%). A prediction for survival would miss very few (6.7%) patients who actually deceased, and of those predicted to decease only 26.3% would actually survive, constituting a useful guidance of early specific treatment for those predicted to decease. The odds ratio (89.6) and relative ratio (24.3) obtained here are higher than those previously reported for individual inflammatory cytokine levels with a maximum hazard ratio of 4.2,[26] genetic markers with maximum odds ratio of 2.5,[30] or chest radiography with maximum accuracy of 74%.[31] Our results emphasize the importance of cytokine combination profiling in assessing severity and mortality.

Conclusions

Our results concur with the notion of an overproduction of a distinct set of cytokines following SARS-CoV-2 infection, although not as a unique cytokine storm, but as the occurrence of distinct cytokine profiles associated with respiratory severity in patients with COVID-19. Furthermore, mortality in the different severity groups was also found associated with distinct cytokine production profiles. We therefore advocate improved personalized patient management through cytokine profiling.

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Key messages

- **COVID-19 severity is associated with distinct cytokine profiles, whereas measurement of 6 cytokines at hospital admission predicts mortality.**
- **SARS-CoV-2 antigenemia, associated with mortality in moderate severity patients, correlates with levels of type-I interferon, but not with other proinflammatory cytokines.**
- **These results call for personalized COVID-19 management based on a combined cytokine/viral load profiling.**

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