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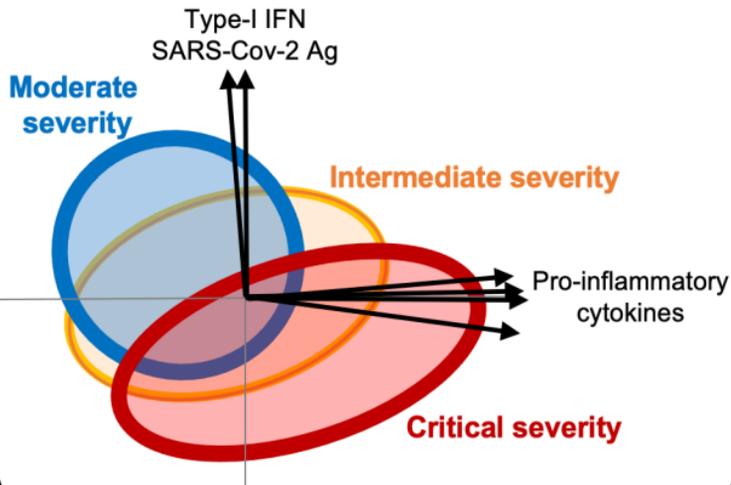
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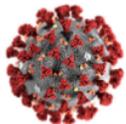
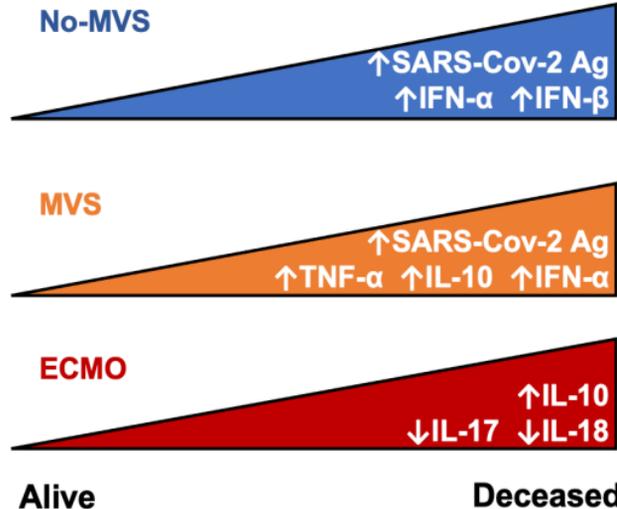
Distinct cytokine profiles associated with COVID-19 severity and mortality

Distinct serum cytokine combinations classify COVID-19 severity at hospital admission



Initial cohort: N=115; validation cohort: N=86

Differential prediction of mortality by cytokine profiles and viral antigen (Ag) levels



ECMO, Extracorporeal membrane oxygenation

MVS or No-MVS, mechanical ventilatory support or not



1 **Distinct cytokine profiles associated with COVID-19 severity and mortality**

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56

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58 All the authors have no conflict of interest to declare.

59

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62 **Abstract**

63 **Background:** Markedly elevated levels of pro-inflammatory cytokines and defective type-I
64 interferon responses were reported in COVID-19 patients.

65 **Objective:** This study aimed to determine whether particular cytokine profiles are associated
66 with COVID-19 severity and mortality.

67 **Methods:** Cytokine concentrations and SARS-CoV-2 antigen were measured at hospital
68 admission in serum of symptomatic COVID-19 patients (N=115), classified at hospitalization
69 into three respiratory severity groups: no need for mechanical ventilatory support (No-MVS),
70 intermediate severity requiring mechanical ventilatory support (MVS) and critical severity
71 requiring extracorporeal membrane oxygenation (ECMO). Principal component analysis was
72 used to characterize cytokine profiles associated with severity and mortality. The results were
73 thereafter confirmed in an independent validation cohort (N=86).

74 **Results:** At time of hospitalization, ECMO patients presented a dominant pro-inflammatory
75 response with elevated levels of tumor necrosis factor-alpha (TNF- α), interleukin (IL)-6, IL-8
76 and IL-10. In contrast, an elevated type-I interferon response involving IFN- α and IFN- β was
77 characteristic of No-MVS patients, whereas MVS patients exhibited both profiles. Mortality
78 at one month was associated with higher levels of pro-inflammatory cytokines in ECMO
79 patients, higher levels of type-I interferons in No-MVS patients and their combination in
80 MVS patients, resulting in a combined mortality prediction accuracy of 88.5% (Risk Ratio
81 24.3, $p < 0.0001$). SARS-CoV-2 antigen levels correlated with type-I interferon levels and
82 were associated with mortality, but not with pro-inflammatory response or severity.

83 **Conclusion:** Distinct cytokine profiles are observed in association with COVID-19 severity
84 and are differentially predictive of mortality according to oxygen support modalities. These
85 results warrant personalized treatment of COVID-19 patients based on cytokine profiling.

86

87 Key Messages (2-3 short bullet points)

- 88 • COVID-19 severity is associated with two distinct cytokine profiles, whereas
89 measurement of six cytokines at hospital admission predicts mortality.
- 90 • SARS-CoV-2 antigenemia, associated with mortality in moderate severity patients,
91 correlates with levels of type-I interferon, but not of other pro-inflammatory cytokines
- 92 • These results call for personalized COVID-19 management based on a combined
93 cytokine/viral load profiling

94 Capsule Summary

95 Distinct cytokine profiles are observed in association with COVID-19 severity and are
96 differentially predictive of mortality in different oxygen support modalities. These results
97 warrant personalized treatment of COVID-19 patients based on cytokine profiling.

98 Key Words (up to 10)

99 COVID-19, serum cytokines, type-I IFNs, respiratory severity, mortality, principal
100 component analysis.

101 Abbreviations

102 Ag, antigen; ALQ, above upper limit of quantification; COVID-19, coronavirus disease 2019;
103 fcc, cytokine combination function; ECMO, extracorporeal membrane oxygenation; IFN,
104 interferon; IL, interleukin; MVS, mechanical ventilatory support; PCA, principal component
105 analysis; SAPS-II, simplified acute physiology score; SARS-CoV-2, severe acute respiratory
106 syndrome coronavirus 2; TNF, tumor necrosis factor.

107

108 Introduction

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

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

123 In the present study, we used highly sensitive, classical or digital, multiplex enzyme-linked
124 immunosorbent assay technologies, to analyze combined cytokine production profiles in
125 serum of patients with COVID-19, at the time of hospital admission. Thereafter, we used a
126 non-supervised bioinformatics approach to identify the cytokine combinations that represent a
127 reliable biomarker of COVID-19-related pulmonary severity and/or mortality.

128

129 **Methods**

130 **Patients and samples**

131 This prospective study was performed at Pitié-Salpêtrière hospital in Paris and approved by
132 the local ethical committee, *Comité d’Ethique de la Recherche* (CER-SU) of Sorbonne
133 University, #CER-2020-21 and -31. Patients with real-time reverse-transcriptase-polymerase-
134 chain reaction (RT-PCR)-confirmed SARS-CoV-2 RNA presence in their nasopharyngeal
135 swab, and pulmonary manifestations, were included after admission to intensive care units
136 (ICU) or to the Internal Medicine department.

137 A total of 115 COVID-19 patients (demographic and clinical characteristics in **Table 1** and
138 **Table E1**) were enrolled between March 17 to May 11, 2020 in the initial cohort. Patients
139 were divided at day of hospitalization in 3 groups according to severity of pulmonary
140 involvement: *i*) moderate severity treated with no oxygen support, nasal cannula or oxygen
141 mask (No-MVS, N=34), *ii*) intermediate severity requiring Mechanical Ventilatory Support
142 (MVS, N=50), and *iii*) critical respiratory failure requiring Extracorporeal Membrane
143 Oxygenation (ECMO, N=31). At 1 month after admission, 7 (20.6%), 19 (38%) and 7
144 (22.6%) patients were deceased in the No-MVS, MVS and ECMO groups, respectively. In
145 addition, healthy SARS-CoV-2-negative individuals (N=10) were included as controls.

146 A validation cohort included 86 COVID-19 patients (clinical characteristics in **Table E2**)
147 enrolled between June 28 to November 23, 2020. Patients were classified into respiratory
148 severity groups as in the initial cohort (No-MVS: N=10, MVS: N=58 and ECMO: N=18).
149 New therapeutic guidelines(6) lead to a decrease in mortality: No-MVS: 0 (0%), MVS: 16
150 (27.6%) and ECMO: 4 (22.2%). In particular, all patients in validation cohort received
151 glucocorticoids treatment.

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

157 **Immunoassays**

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

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

162 Procedures and specificity of the immunoassays used in this work are detailed in the Online
163 Repository Methods section.

164 **Statistical and bioinformatics analysis**

165 Principal component analysis (PCA) was used to determine the cytokine combinations
166 associated with severity and mortality. Statistical significance of differences between groups
167 was assessed using the non-parametric Mann-Whitney and Kruskal-Wallis tests for
168 continuous variables and Fisher-Exact test for frequencies. Correlations were assessed by the
169 non-parametric Spearman test. Analyses were performed with SPSS, GraphPad-Prism and R.
170 Two-sided P-value <0.05 was considered statistically significant. ns: non-significant; *
171 p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

172 For detailed methods, please see the Methods section in this article's Online Repository at
173 www.jacionline.org.

174 **Results**

175 **Patients' severity and mortality**

176 COVID-19 patients in the initial cohort (N=115) were classified into three respiratory severity
177 groups: no need for mechanical ventilatory support (No-MVS), intermediate severity
178 requiring MVS and critical severity requiring extracorporeal membrane oxygenation
179 (ECMO). Mortality at one month after admission (28.7%) was not different between the three
180 severity groups (p=0.23). The simplified acute physiology score (SAPS-II, median 36, IQR:
181 26 – 49), which classifies disease severity for patients admitted to intensive care units(7),
182 validated our respiratory severity classification, with median SAPS-II of 26, 35 and 52 for
183 No-MVS, MVS and ECMO groups respectively, p<0.001 (Figure E1). Patients' SAPS-II
184 scores were associated with mortality only in the MVS group (Figure E1).

185 **Distinct cytokine profiles associated with COVID-19 severity**

186 Concentrations of 12 cytokines were measured in serum from the COVID-19 patients at
187 hospital admission, and in healthy SARS-CoV-2-negative subjects (Figure E2). As expected,
188 principal component analysis (PCA) emphasized important overall cytokine profile
189 differences (p<0.001) between COVID-19 patients and healthy controls, but also underlined
190 the heterogeneity of the COVID-19 group (Figure 1A).

191 To elucidate the association of serum cytokine levels with COVID-19 severity, we performed
192 non-supervised PCA among COVID-19 patients only, highlighting two distinct cytokine
193 combinations (Figure 1B). Pro-inflammatory cytokines, including TNF- α , IL-6, IL-8, and IL-
194 1 β , as well as IL-10, IL-22, mainly contributed to the first principal component (PC1), while
195 the type-I interferon anti-viral cytokines IFN- α and IFN- β had an orthogonal contribution on
196 the second principal component (PC2). Strikingly, these two distinct cytokine combinations
197 were differentially associated with COVID-19 respiratory severity, with 96.4% of ECMO

198 patients and 96.6% of No-MVS patients appropriately segregated by the separatrix line
199 ($p < 0.0001$).

200 Taking into account only the cytokines with the greatest contribution to the principal
201 components (Figure 1B), two cytokine combination functions (f_{CC}) were constructed: f_{CC-}
202 $INFLAM$, based on the pro-inflammatory cytokines: TNF- α , IL-6, IL-8 and IL-10, and f_{CC-}
203 $IFNI$, based on the type-I interferons IFN- α and IFN- β (see further information in article's Online
204 Repository). These two cytokine combinations were distinctively associated with severity:
205 $f_{CC-}INFLAM$ values were higher in ECMO patients ($p < 0.0001$: Figure 1C), while $f_{CC-}IFNI$ values
206 were higher in No-MVS patients ($p < 0.0001$: Figure 1D), in a mutually exclusive manner.

207 The cytokine profiles of the MVS patients were scattered in between those of the two other
208 groups (Figure 1B), showing intermediate levels of both $f_{CC-}INFLAM$ and $f_{CC-}IFNI$ (Figures 1C-
209 D). Indeed, analysis by k-means clustering revealed that MVS patients could be divided into
210 three sub-groups ($p < 0.01$) with different combinations of elevated $f_{CC-}INFLAM$ and/or $f_{CC-}IFNI$
211 values (Figure E3).

212 Repeating the same analysis with severity groups defined by SAPS-II scores showed the same
213 association of $f_{CC-}INFLAM$ and $f_{CC-}IFNI$ with COVID-19 severity (Figure E1B). Moreover, these
214 results were confirmed for patients with intermediate time lapse from symptoms onset (Figure
215 E4), for whom there was no difference in this time lapse between the groups (Table 1) and no
216 variation of cytokine levels as function of time from symptoms' onset (Figure E4). Logistic
217 regression analysis taking into account all baseline factors (Table E1) confirmed that f_{CC-}
218 $INFLAM$ and $f_{CC-}IFNI$ were the only factors remaining associated with severity in a statistically
219 significant manner after multi-factorial correction.

220 Furthermore, our results were confirmed in an independent validation cohort (N=86). PCA of
221 the validation cohort patients alone (Figure E5A), or both cohorts combined (Figure E5B),
222 showed again segregation of ECMO patients having higher levels of pro-inflammatory

223 cytokines and No-MVS patients having higher levels of type-I interferon response.
224 Accordingly, the values of the cytokine combination functions $f_{CC-INFLAM}$ and $f_{CC-IFNI}$ were
225 mutually exclusively, and significantly different between the respiratory severity groups, for
226 the validation cohort alone (Figures E5C and E5E) or for both cohorts together (Figures E5D
227 and E5F).

228 **COVID-19 mortality risk is associated with distinct cytokine profiles**

229 To elucidate the association of serum cytokine levels at time of hospitalization with COVID-
230 19 mortality one month later, we performed PCA for each oxygen support modality
231 separately, since PCA for all patients together did not show separation between alive and
232 deceased patients. Mortality was differentially associated with distinct cytokine combinations
233 in each group. In ECMO patients, mortality was associated with elevated levels of pro-
234 inflammatory cytokines (in particular IL-10, IL-8, IL-6 and TNF- α), as well as with decreased
235 levels of IL-17A and IL-18 (Figure 2C). On the other hand, in No-MVS patients elevated
236 levels of IFN- α and IFN- β , but not pro-inflammatory cytokines, were associated with
237 mortality (Figure 2A). Lastly, in MVS patients with SAPS-II ≥ 35 (median of the MVS group,
238 below which only 4% of MVS patients deceased), mortality was associated with the
239 combination of elevated levels of both pro-inflammatory cytokines (in particular TNF- α and
240 IL-10) and IFN- α (Figure 2B).

241 By selecting the cytokines most associated with mortality according to the PC factors (Figures
242 2A-C), we defined a cytokine combination function for each group: f_{No-MVS} based on IFN- α
243 and IFN- β for No-MVS patients (Figure 2D), f_{MVS} based on TNF- α , IL-10 and IFN- α for
244 MVS patients with SAPS-II ≥ 35 (Figure 2E), and f_{ECMO} , based on IL-10, IL-17A and IL-18
245 for ECMO patients (Figure 2F). The magnitude of each of these cytokine combinations was
246 significantly higher in deceased as compared to alive patients in each corresponding severity
247 group, but not in the other groups. Furthermore, regression analysis with other co-factors such

248 as demographics, past medical history and COVID-19-related treatment (Table E1), showed
249 that f_{No-MVS} , f_{MVS} and f_{ECMO} were the only factors that remained significantly associated with
250 mortality after multi-factorial correction per each group accordingly.

251 The differential association of mortality with the different cytokine combination functions
252 identified in the initial cohort was further confirmed in an independent validation cohort
253 (N=86). PCA for each of the severity groups (No-MVS, MVS and ECMO), using only the
254 cytokines in each cytokine combination function (f_{No-MVS} , f_{MVS} and f_{ECMO}) accordingly,
255 separated deceased versus alive patients for the initial and validation cohorts together (Figures
256 E6A-C). Furthermore, values of f_{No-MVS} , f_{MVS} and f_{ECMO} from both cohorts together showed a
257 significant difference between alive versus deceased patients, but only for the cytokine
258 combination function of the corresponding group (Figures E6D-F).

259 Next, we set appropriate thresholds (selected to optimize accuracy of prediction, Figure 2) for
260 each of the cytokine combination functions (f_{No-MVS} , f_{MVS} and f_{ECMO}) and generated the
261 prediction table and scores for each group accordingly (Table 2). The overall mortality of
262 COVID-19 patients at one month after admission could be predicted by measuring circulating
263 levels of only six cytokines, as well as the SAPS-II score for the MVS group, with an
264 accuracy of 88.5%, a sensitivity of 93.3% and a specificity of 86.5% (Risk Ratio 24.3, Odds
265 Ratio 89.6, $p < 0.0001$, Table 2).

266 To investigate the robustness of the predictions, we plotted receiver operating characteristic
267 (ROC) curves of mortality outcome comparing the three cytokine combination functions,
268 SAPS and age (Figure E7), as well as all individual cytokine levels (Figure E8) in each of the
269 respiratory severity groups. In each group, the corresponding prediction function always
270 represented the largest area under the curve (AUC). Moreover, to evaluate the robustness of
271 the differential predictions, we calculated the relative sensitivity (rSens) and relative
272 specificity (rSpec) of the corresponding function for each group and compared them with the

273 two other functions (Table E4). The combined relative predictive score ($rSens*rSpec$) was
274 always larger than 1.6, indicating that each function indeed best predicted mortality for the
275 corresponding group.

276 **High viral loads associated with mortality in No-MVS and MVS, but not in ECMO** 277 **patients**

278 SARS-CoV-2 nucleocapsid (N)-antigen levels were measured by ELISA (8) in 179 patients
279 on the same day as the cytokine measurement. However, in 32 patients with sampling later
280 than 14 days after symptoms' onset, antigen levels were significantly lower ($p<0.001$). Thus,
281 for the measurement of SARS-CoV-2 antigen only the 147 patients sampled up to 14 days
282 after symptoms' onset were considered.

283 Viral load was not associated with COVID-19 severity; neither as defined by oxygen support
284 modality groups, nor by SAPS-II scores (Figure 3). However, mortality one month after
285 hospitalization was associated with higher levels of antigen (Figure 3A) in No-MVS patients
286 ($p=0.002$) and MVS patients ($p=0.008$), but not in ECMO patients.

287 **Viral load is correlated with type-I interferon response but not with inflammatory** 288 **cytokines**

289 SARS-CoV-2 antigen levels were significantly correlated with the type-I interferon cytokine
290 combination function $fcc-IFN1$ (Figure 4) in No-MVS patients ($R=0.79$, $p<0.001$) and in
291 ECMO patients ($R=0.57$, $p=0.002$), as well as with $IFN-\alpha$ and $IFN-\beta$ individually, but not
292 with $IFN-\gamma$. However, in intermediate severity MVS patients, antigen levels were not
293 correlated with those of $IFN-\alpha$ and $IFN-\beta$ individually, nor with the $fcc-IFN1$ cytokine
294 combination, but were correlated with $IFN-\gamma$ levels instead ($R=0.45$, $p<0.001$). On the other
295 hand, inflammatory cytokine levels, either individually or in the cytokine combination
296 function $fcc-INFLAM$, were not significantly correlated with viral load. Interestingly, deceased

297 No-MVS and MVS patients (Figures 4A-B), but not ECMO patients (Figure 4C), had both
298 higher levels of antigen and type-I interferons.

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

300 Major baseline demographic parameters associated with COVID-19 severity or mortality(1)
301 (gender, age, smoking, BMI, obesity and overweight) were neither significantly associated
302 with SARS-CoV-2 viral load nor with any of the cytokine levels or their combinations. Also,
303 most baseline clinical conditions (cancer, immunodeficiency, chronic pulmonary disease,
304 asthma, COPD, chronic liver disease, chronic neurologic disease) did not show a significant
305 association with SARS-CoV-2 antigen levels or with any of the cytokine levels or their
306 combinations. However, we found that chronic kidney disease was significantly ($p=0.002$)
307 associated with higher SARS-CoV-2 antigen and IFN- α ($p<0.001$) levels. Patients with
308 diabetes, cardiovascular disease, and hypertension, also showed a trend for higher viral load
309 and IFN- α levels, but this was not significant when considering the occurrence of kidney
310 disease.

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

314

315 Discussion

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

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

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

339 The association of enhanced serum cytokine levels with mortality does not necessarily imply
340 cytokine-related toxicity. While IL-6, IL-8 or TNF- α are known to have deleterious pro-
341 inflammatory effects, IL-10 is considered to mainly dampen inflammation(9), although its
342 expression levels were found to be associated with higher mortality rates in patients requiring
343 MVS and ECMO. These results do not point to a causal effect but to an association of the
344 cytokine profiles with mortality. Elevated levels of other circulating cytokines were also
345 associated with a favorable outcome of the disease, such as high IL-17A and IL-18 levels in
346 ECMO patients (Figure 2C and E8), possibly in relation with their protective role in anti-
347 microbial responses(10, 11).

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

357 The results from the present study corroborate previous reports showing that some critically
358 severe COVID-19 patients are characterized by defective type-I IFN responses (5, 15, 16).
359 Indeed, we recently contributed to report patients with undetectable systemic concentrations
360 of IFN- α , either in the context of genetic inborn errors of cytokine-mediated immunity(17), or
361 dependent on the presence of anti-cytokine auto-antibodies(18). Nevertheless, IFN- α was
362 detectable at high levels within the first seven days after the onset of symptoms also in severe
363 patients (Figure E2 and E4). Similarly, while levels of IFN- β were low in many COVID-19

364 patients, possibly due to a lower sensitivity of detection (Table E3), they were nevertheless
365 not systematically undetectable in severe patients (Figure E2), contrary to what has been
366 previously suggested(16, 19). We show that mortality in No-MVS patients is rather associated
367 with exacerbated type-I IFN responses. While it is possible that elevated serum interferon
368 levels would result from down-stream signaling defects in the type-I IFN pathway(20),
369 previous results suggest that an actively persisting type-I IFN response is associated with
370 immunopathology(21, 22). Of note, an elevated type-I interferon response in these patients
371 was highly correlated with elevated viral load (Figure 4), and thus could simply reflect an
372 appropriate response to viral replication. Although we did not find evidence that an
373 exacerbated type-I interferon response was related to cardiologic (or other) specific
374 complications in No-MVS and MVS patients (Tables E1 and E2), it should be emphasized
375 that mortality in these groups was associated with the highest type-I interferon levels.

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

384 Our findings could also explain why the administration of glucocorticoids, in particular
385 dexamethasone, which decreases blood levels of many cytokines, was successful in both
386 patients receiving invasive mechanical ventilation (MVS) and those receiving oxygen without
387 invasive mechanical ventilation, corresponding to part of No-MVS patients(6, 27). Because
388 mortality in these patients was found to be strongly associated with high levels of type-I IFN,

389 the modulation of secretion and/or activity of the latter cytokines could be related to
390 corticosteroid efficacy(28).

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

397 Associations of cytokine combination functions with COVID-19 severity and mortality were
398 confirmed in an independent, second pandemic wave validation cohort. It should be noted that
399 these patients had all received corticosteroids at the time of sampling (in contrast to the
400 patients of the initial cohort). The observed associations hold both with and without
401 corticosteroid treatment. The mortality prediction table presented here was calculated based
402 only on the initial cohort results, since those were measured at day of hospitalization, unlike
403 in the validation cohort. ROC curve analysis and the 95% CI of the odds ratios show the
404 robustness of the mortality predictions per severity group. Altogether, based on 6 cytokines
405 measured at day of hospitalization we obtained a highly accurate (88.5%) mortality prediction
406 with 93.3% sensitivity and 86.5% specificity (PPV=73.7% and NPV=97%). A prediction for
407 survival would miss very few (6.7%) patients that actually deceased, and of those predicted to
408 decrease only 26.3% would actually survive, constituting a useful guidance of early specific
409 treatment for those predicted to decrease. The odds ratio (OR=89.6) and relative ratio
410 (RR=24.3) obtained here are higher than those previously reported for individual
411 inflammatory cytokine levels with maximum HR of 4.2(26), genetic markers with maximum
412 OR of 2.5 (30) or chest radiography with maximum accuracy of 74% (31). Our results

413 emphasize the importance of cytokine combination profiles in assessing severity and
414 mortality.

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

421 **Author Contributions:**

422 KD and GG designed the study. KD, PQ, MG, SM, DS, SB and AUN compiled the data and
423 performed data analysis. AG, CEL, MS, JM, AB, LLG, SD, EBS, AM, CP, ZA, GV and GG
424 provided patient sample access. GG, AG, BC, CC and AGM provided financial support. KD,
425 HY and GG wrote the initial draft. KD, PQ, MG, HY, CTH, AUN and GG edited the
426 manuscript. All authors revised and contributed to the manuscript.

427

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435

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527 Prognostication of Patients with COVID-19 from the Emergency Department. *Radiology:*
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529

530 **Table 1. Demographics, baseline characteristics and clinical outcomes of 115 COVID-19**
 531 **patients in the 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 (83.3)	83 (72.2)	0.36
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	-	-	31 (100)	31 (27)	
Time from onset of symptoms to admission / sample					
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)	0.03
Type 2 diabetes	11 (32.4)	19 (38)	12 (38.7)	42 (36.5)	0.83
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)	0.96
Any condition*	28 (82.4)	41 (82)	28 (90.3)	97 (84.3)	0.57
Clinical outcome at 1 month – no. (%)					
Discharged	27 (79.4)	30 (60)	20 (64.5)	77 (67)	0.17
Remained in hospital	0 (0)	1 (2)	4 (12.9)	5 (4.3)	0.25
Deceased	7 (20.6)	19 (38)	7 (22.6) [£]	33 (28.7) [£]	0.23
Median length of stay ^Ω , days (IQR)	10 (6 – 16)	10 (7 – 22)	32 (25 – 55)	13 (7 – 29)	< 0.001

532 [‡] Kruskal-Wallis test for continuous variables, Fisher exact test for discrete variables.

533 *Including hypertension, diabetes, obesity, cancer, arterial thromboembolism, venous

534 thromboembolism, systemic autoimmune disease, HIV infection, solid organ transplant,

535 chronic pulmonary disease, chronic liver disease, chronic kidney disease, chronic neurologic
536 disease and cardiovascular disease.

537 £2 additional patients died later than day 30 of hospitalization (at day 46 and day 50).

538 ^ΩAs of June 18, 2020.

539 ECMO, Extracorporeal Membrane Oxygenation; SAPS II, Simplified Acute Physiology

540 Score II; SOFA, Sequential Organ Failure Assessment.

541 Bold values indicate statistical significance.

542 **Table 2: Prediction of COVID-19 mortality at 1 month from admission using cytokine**
 543 **combinations measured at hospital admission.**

	Mortality Prediction ¹			
Group	No-MVS	MVS	ECMO	ALL
Predictor	$f_{\text{NoMVS}}(\text{IFN-}\alpha, \text{IFN-}\beta)$	$f_{\text{MVS}}(\text{SAPS}, \text{TNF-}\alpha, \text{IL-10}, \text{IFN-}\alpha)$	$f_{\text{ECMO}}(\text{IL-10}, \text{IL-17A}, \text{IL-18})$	$f_{\text{NoMVS}}, f_{\text{MVS}}, f_{\text{ECMO}}$
N ²	29	47	28	104
Mortality	24.1%	36.2%	21.4%	28.9%
Accuracy	86.2%	91.5%	85.7%	88.5%
NPV ³	100%	93.3%	100%	97.0%
PPV ³	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 ⁴	> 29.8	105	> 25.5	89.6
[95% CI]	[2.8, 315.6]	[13.4, 822.1]	[2.4, 275.7]	[18.4, 435.8]
RR ⁴	> 11.5	13.2	> 10.8	24.3
[95% CI]	[1.6, 81.0]	[3.4, 51.1]	[1.5, 77.5]	[6.1, 96.5]
p-value ⁵	0.0002	<0.0001	0.0006	<0.0001

1) Prediction of mortality was performed using following functions:

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

MVS: $\text{SAPS-II} \geq 35$ & $f_{\text{MVS}} = (0.474 * \text{Log}(\text{TNF-}\alpha) + 0.444 * \text{Log}(\text{IL-10}) + 0.194 * \text{Log}(\text{IFN-}\alpha)) > 0.5$
 where $\text{SAPS-II} = 35$ is the median for MVS patients.

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

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

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

2) Some of the numbers are lower than those in Table 1 because of missing cytokine data in few patients

3) NPV: Negative Predictive Value; PPV: Positive Predictive Value

5) OR: Odds Ratio; RR: Risk Ratio; CI: Confidence Interval

6) Statistical significance of the prediction was assessed by the Fisher-Exact test.

545 **Figure legends**

546 **Figure 1: Distinct cytokine profiles associated with COVID-19 respiratory severity. A)**
 547 PCA of twelve serum cytokines measured in COVID-19 patients and controls. **B)** PCA, and
 548 PC factors (table), of the eight cytokines most contributing to inter-patient variation in
 549 COVID-19 patients, segregates the patients by respiratory severity groups. Ellipses represent
 550 the 68% confidence interval of patient distribution in each group. Levels of the ensuing
 551 cytokine combination functions $f_{CC-INFLAM}$ (**C**), for inflammatory cytokines derived from PC1,
 552 and $f_{CC-IFNI}$ (**D**), for type-I interferons derived from PC2, are depicted for each respiratory
 553 severity group and the controls. Initial PCA with all 12 cytokines measured in the COVID-19
 554 patients had shown that IFN- γ , GM-CSF, IL-17A and IL-18 either contribute less to the
 555 variation between patients and/or have a mixed contribution to PC1 and PC2. PC1 and PC2
 556 contributed most of the variation between patients (67.3%), while higher PC dimensions had
 557 lower contributions (PC3=9.7%, PC4=6.6%) and did not contribute to separation of patients
 558 by severity.

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

560 **Figure 2: Distinct cytokine combinations associated with COVID-19 mortality.** PCAs for
 561 each respiratory severity group: No-MVS (**A**), MVS^(*) (**B**) and ECMO (**C**) segregate
 562 surviving versus deceased patients in association with different cytokine combinations
 563 (corresponding PC factors tables). Levels of the ensuing cytokine combination functions from
 564 the PC factors for each group: f_{No-MVS} (**D**), f_{MVS} (**E**), and f_{ECMO} (**F**) are depicted for surviving
 565 versus deceased patients in each respiratory severity group.

566 (*) MVS with SAPS-II ≥ 35 (median of the MVS patients).

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

568 **Figure 3: SARS-Cov-2 serum viral load association with severity and mortality. A)**
569 SARS-CoV-2 nucleocapsid (N)-antigen levels depicted by respiratory severity group and
570 deceased versus surviving patient per group (No-MVS: 100% ALQ in deceased versus 34.5%
571 ALQ in surviving patients, $p=0.002$; MVS: 86.4% ALQ in deceased versus 53.2% ALQ in
572 surviving patients, $p=0.008$; ECMO: 16.7% ALQ in deceased versus 47.6% ALQ in surviving
573 patients, $p=ns$). **B)** SAPS-II scores depicted as function of N-antigen levels per patient
574 ($R=0.05$, $p=ns$), with deceased versus surviving patients marked.

575 Only patients with a sample taken at most 14 days after symptoms' onset are included in the
576 analysis, because antigen levels were significantly lower in samples taken after 14 days (50%
577 BLD), as compared to until day 14 days (5.4% BLD, $p<0.0001$). For samples until 14 days
578 there was no correlation of N-antigen levels with time from symptoms' onset and no
579 difference between the cohorts.

580 ALQ: above upper limit of quantification; BLD: below limit of detection.

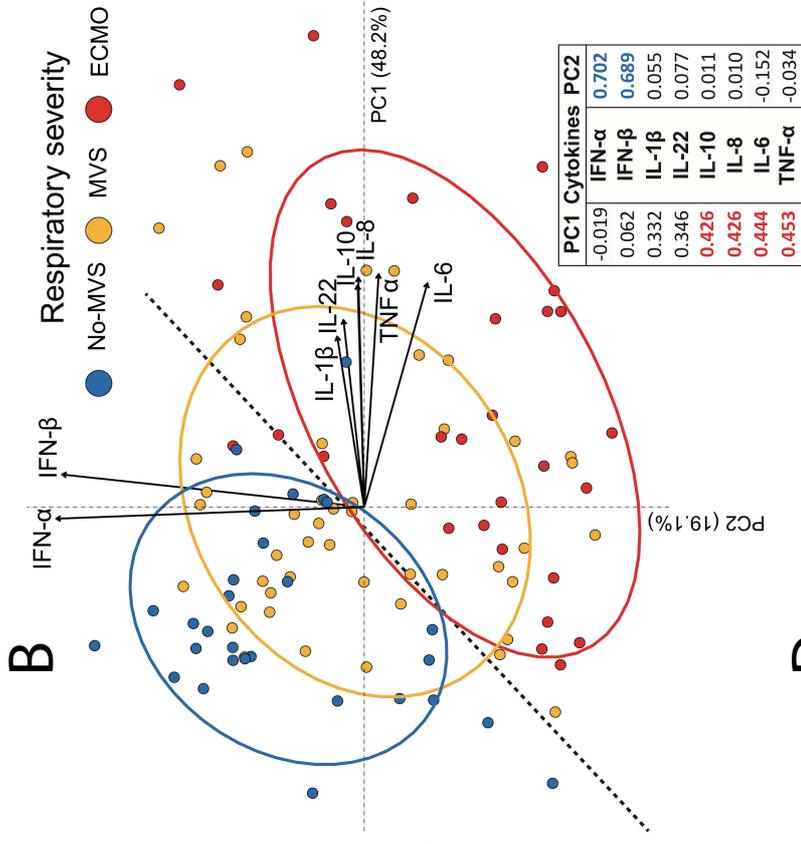
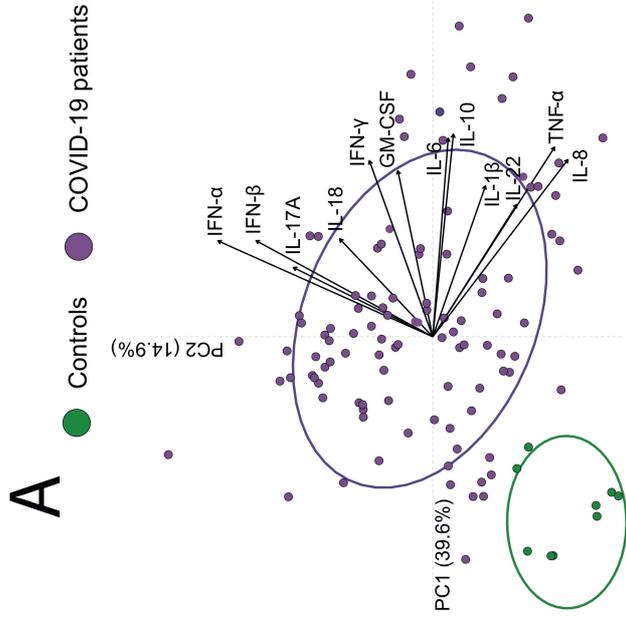
581 ns: non-significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

582 **Figure 4: Correlation between SARS-Cov-2 serum viral loads and cytokine levels.** The
583 cytokine combination functions values for fcc-IFN1 comprising of type-I interferons IFN- α and
584 IFN- β (**A, B, C**) and fcc-INFLAM comprising of the inflammatory cytokines TNF- α , IL-10, IL-
585 6 and IL-8 (**D, E, F**) are plotted as function of SARS-CoV-2 nucleocapsid (N)-antigen levels
586 for each of the respiratory severity groups.

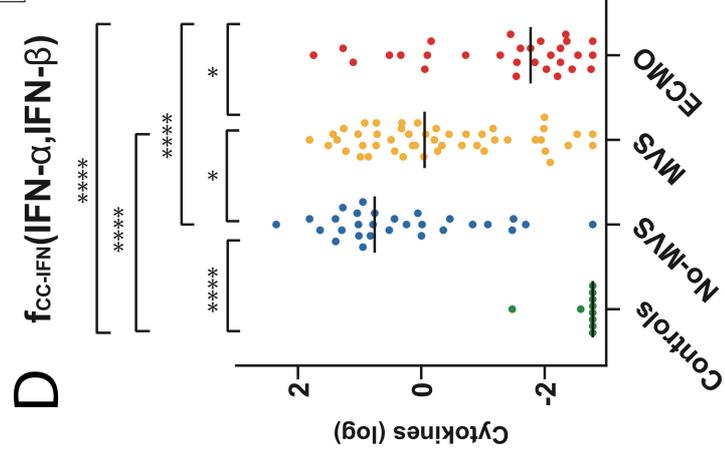
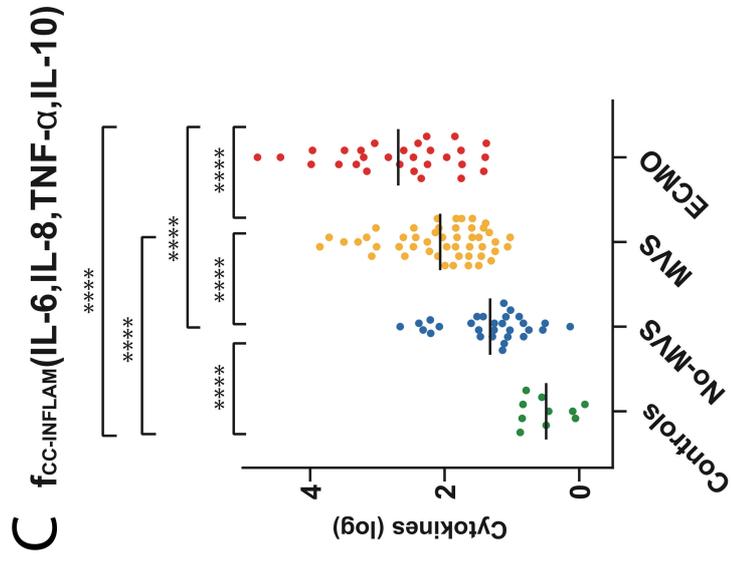
587 Only patients with a sample taken at most 14 days after symptoms' onset are included in the
588 analysis (see Figure 3).

589 ALQ: above upper limit of quantification; BLD: below limit of detection.

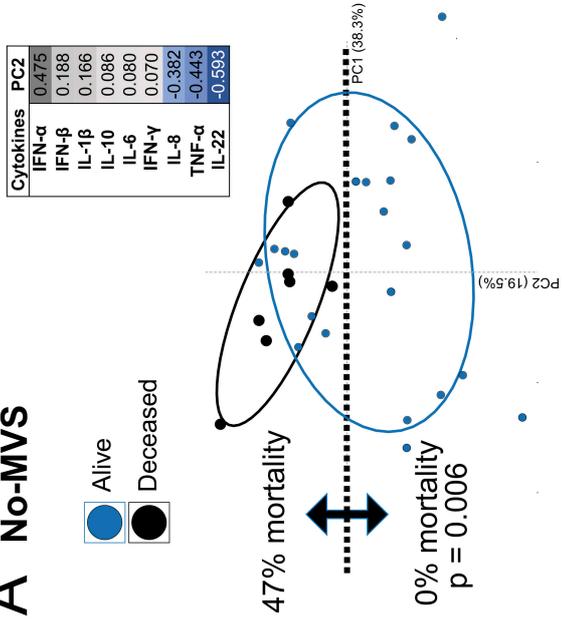
590



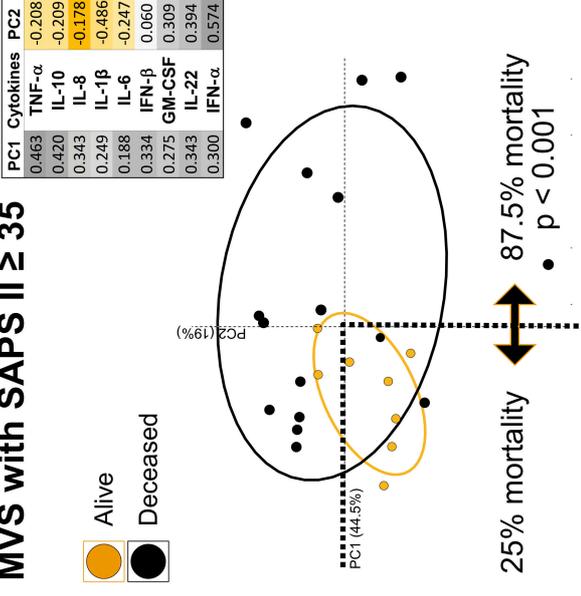
PC1	Cytokines	PC2
-0.019	IFN-α	0.702
0.062	IFN-β	0.689
0.332	IL-1β	0.055
0.346	IL-22	0.077
0.426	IL-10	0.011
0.426	IL-8	0.010
0.444	IL-6	-0.152
0.453	TNF-α	-0.034



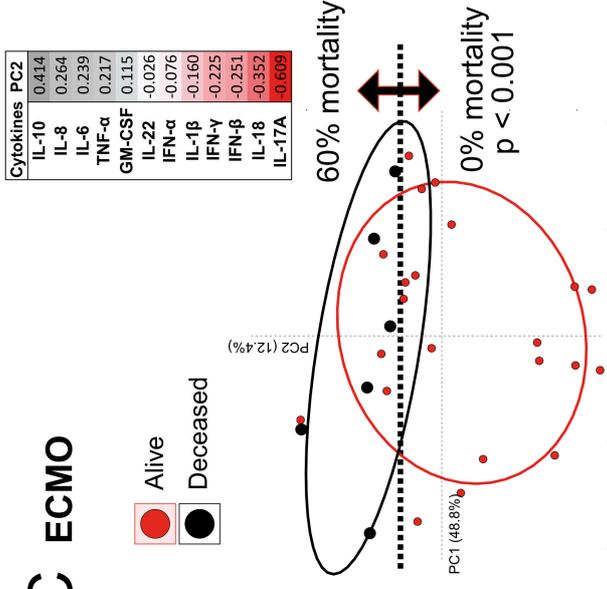
A No-MVS



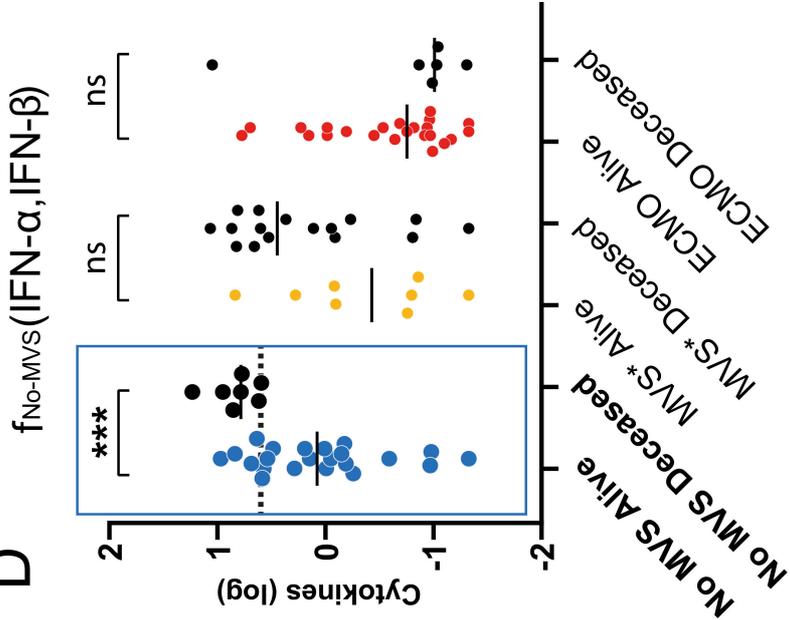
B MVS with SAPS II ≥ 35



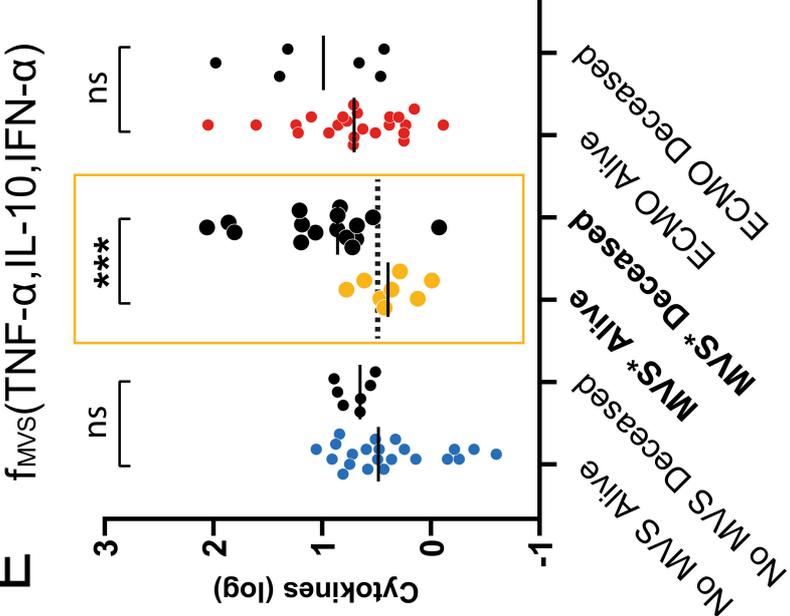
C ECMO



D



E



F

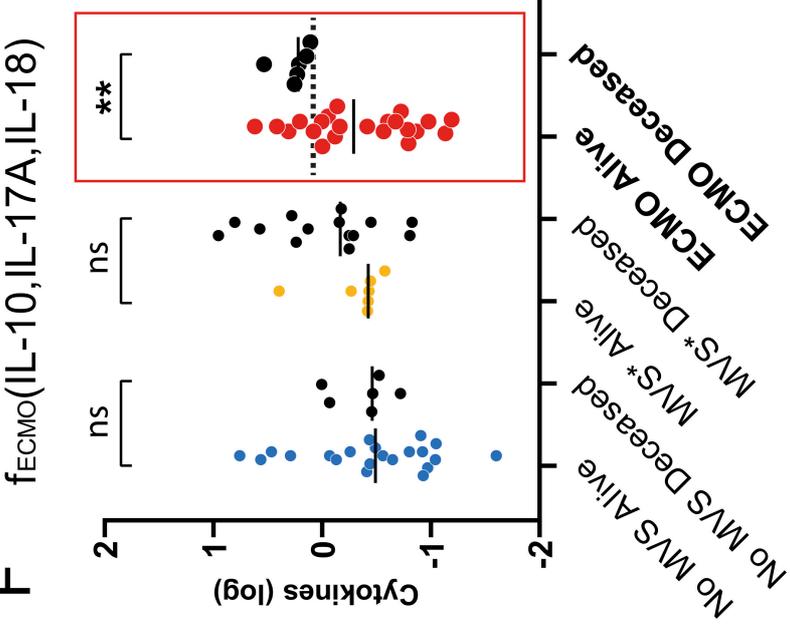
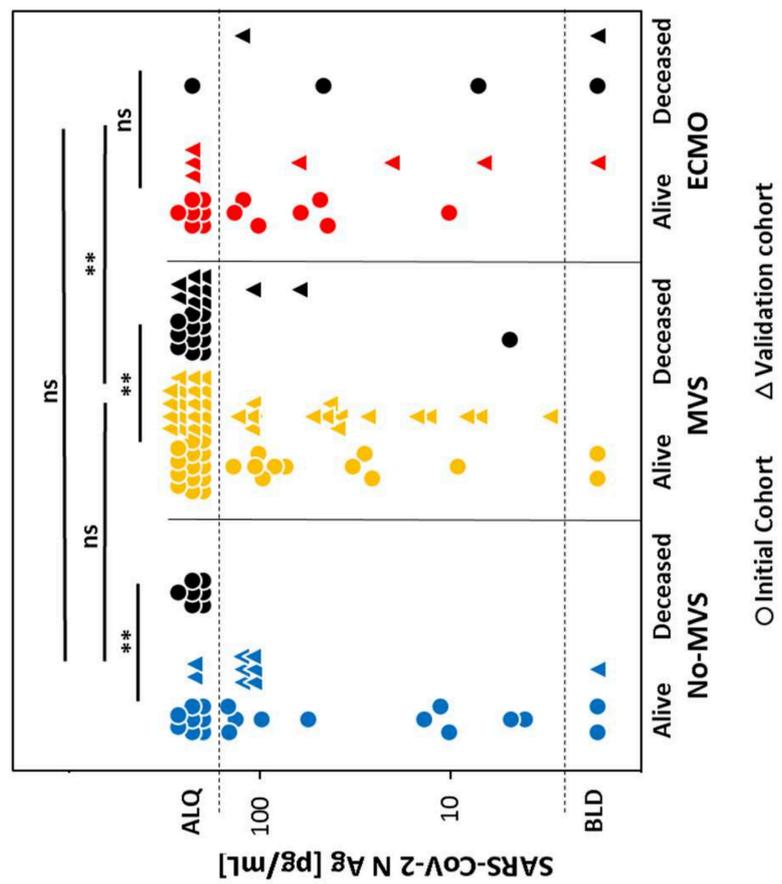


Fig. 3

A



B

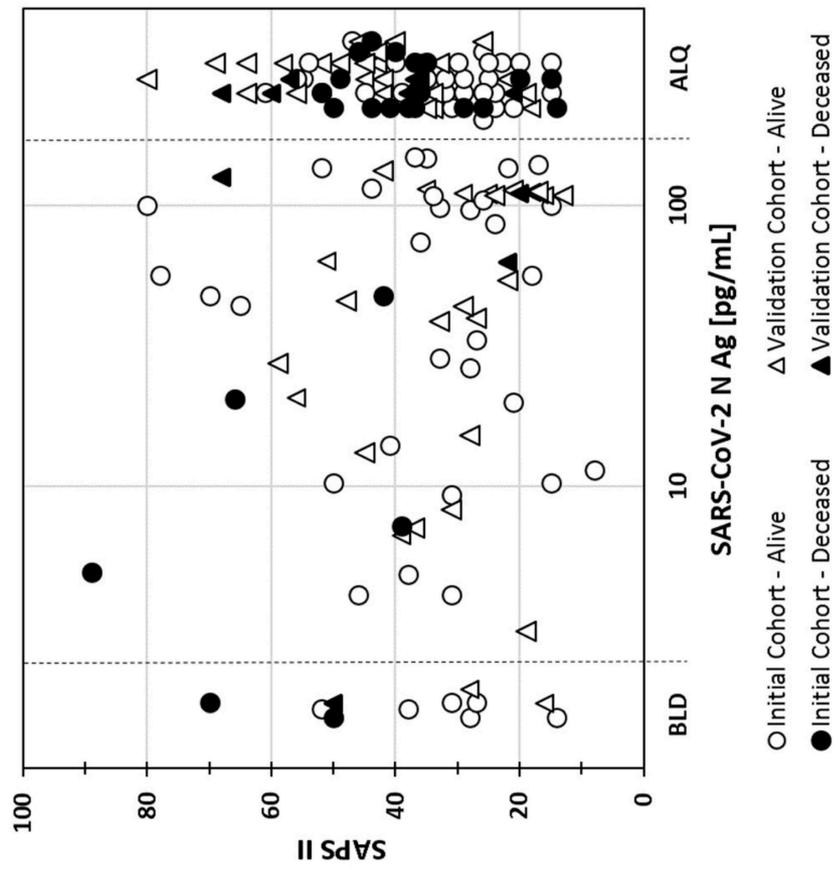
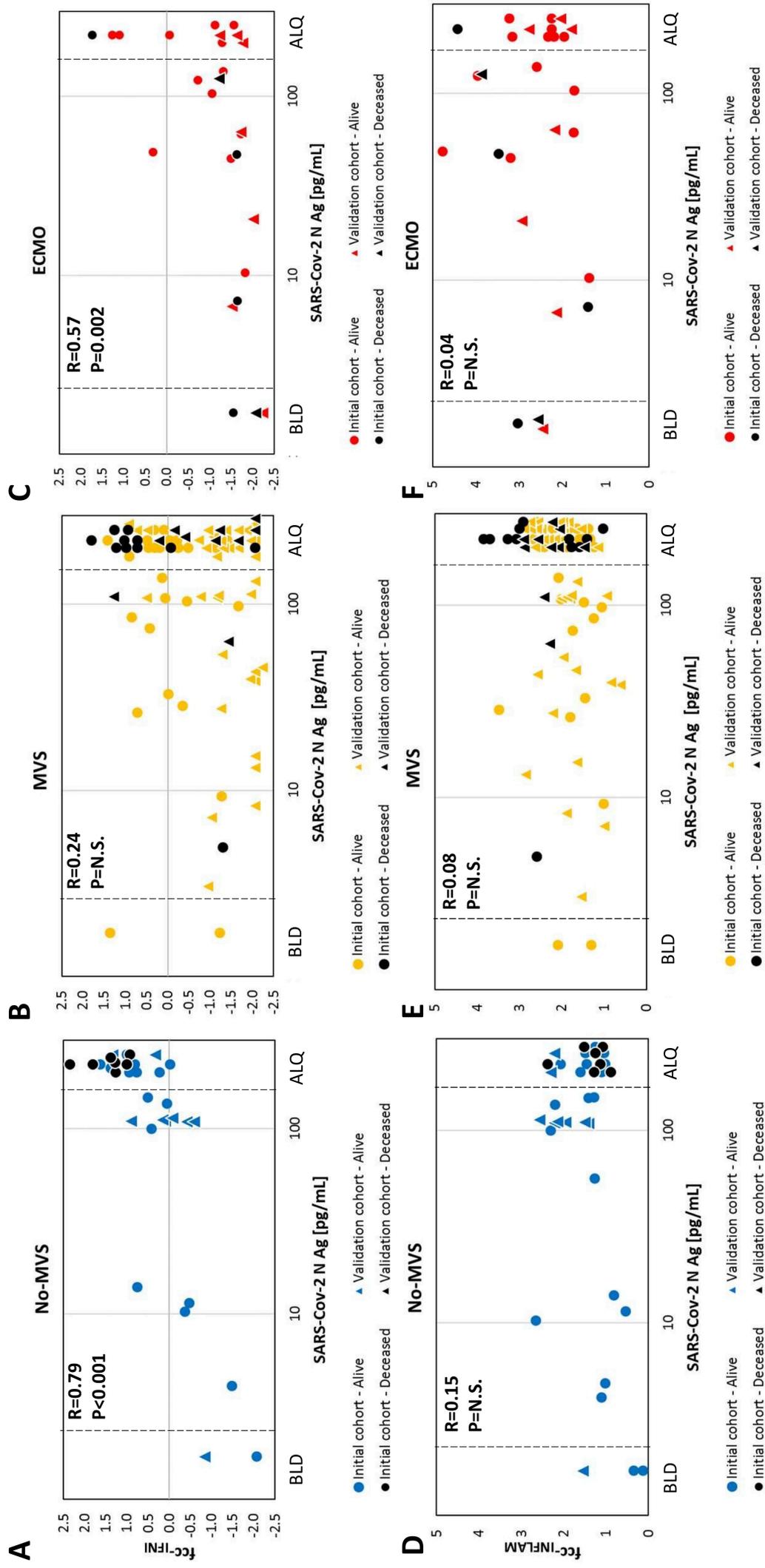


Fig. 4



Distinct cytokine profiles associated with COVID-19 severity and mortality

Online Repository

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-1 β , 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:

For severity classification:

$$f_{cc-INFLAM} = 0.453 * \text{Log}[\text{TNF-}\alpha] + 0.444 * \text{Log}[\text{IL-6}] + 0.426 * \text{Log}[\text{IL-8}] + 0.426 * \text{Log}[\text{IL-10}]$$

$$f_{cc-IFNI} = 0.702 * \text{Log}[\text{IFN-}\alpha] + 0.689 * \text{Log}[\text{IFN-}\beta]$$

For mortality prediction in No-MVS group:

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

For mortality prediction in MVS (SAPS-II \geq 35) group:

$$f_{\text{MVS}} = 0.474 * \text{Log}[\text{TNF-}\alpha] + 0.444 * \text{Log}[\text{IL-10}] + 0.194 * \text{Log}[\text{IFN-}\alpha]$$

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:

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

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:

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

$$\text{MVS (SAPS-II } \geq 35): f_{\text{MVS}} > 0.5$$

$$\text{ECMO: } f_{\text{ECMO}} > 0.1$$

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	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 autoimmune disease.

[‡]2 additional patients died later than day 30 of hospitalisation (at day 46 and day 50).

^ΩAs 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 neurological 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
<i>Smoking habits</i>					
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
<i>Past history of arterial or venous thrombosis</i>					
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 autoimmune disease.

[‡]Four additional patients died later than day 30.

^ΩAs 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-1 β	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 ($f_{\text{No-MVS}}$, f_{MVS} and f_{ECMO}) for each group accordingly versus the predictors for the other 2 groups.

rSens	Mortality predictor ¹		
Group	$f_{\text{No-MVS}}$	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	$f_{\text{No-MVS}}$	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	$f_{\text{No-MVS}}$	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: $f_{\text{No-MVS}} > 0.59$

MVS (SAPS-II ≥ 35): $f_{\text{MVS}} > 0.5$

ECMO: $f_{\text{ECMO}} > 0.1$

* 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; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Ctrl: 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 $\text{fcc-IFN}\gamma$ and being similar to ECMO patients, MVS-2 showing high levels of $\text{fcc-IFN}\gamma$ similarly to No-MVS patients, and MVS-3 group expressing high levels of both fcc-INFLAM and $\text{fcc-IFN}\gamma$. **B-C)** Levels of the cytokine combination functions fcc-INFLAM and $\text{fcc-IFN}\gamma$, 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:

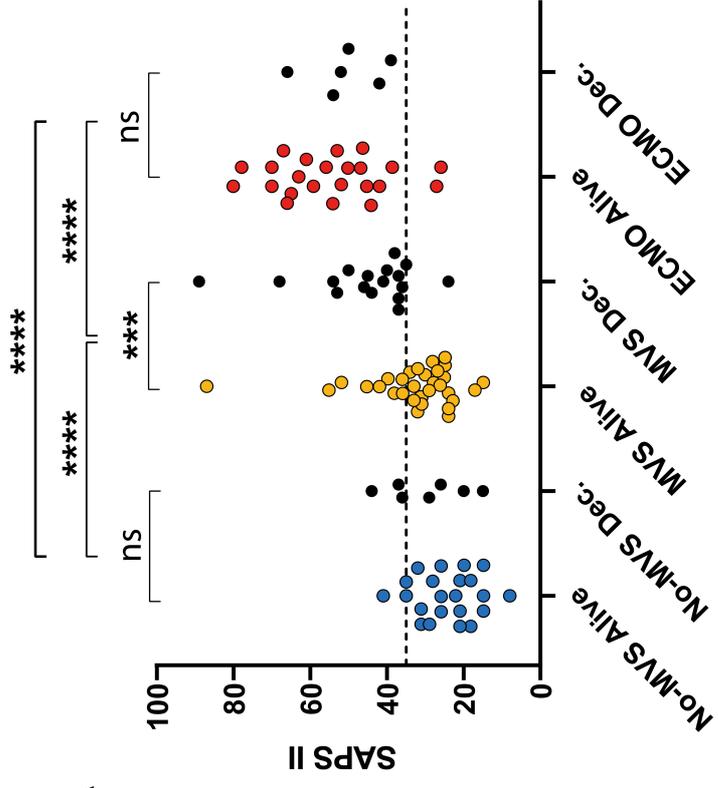
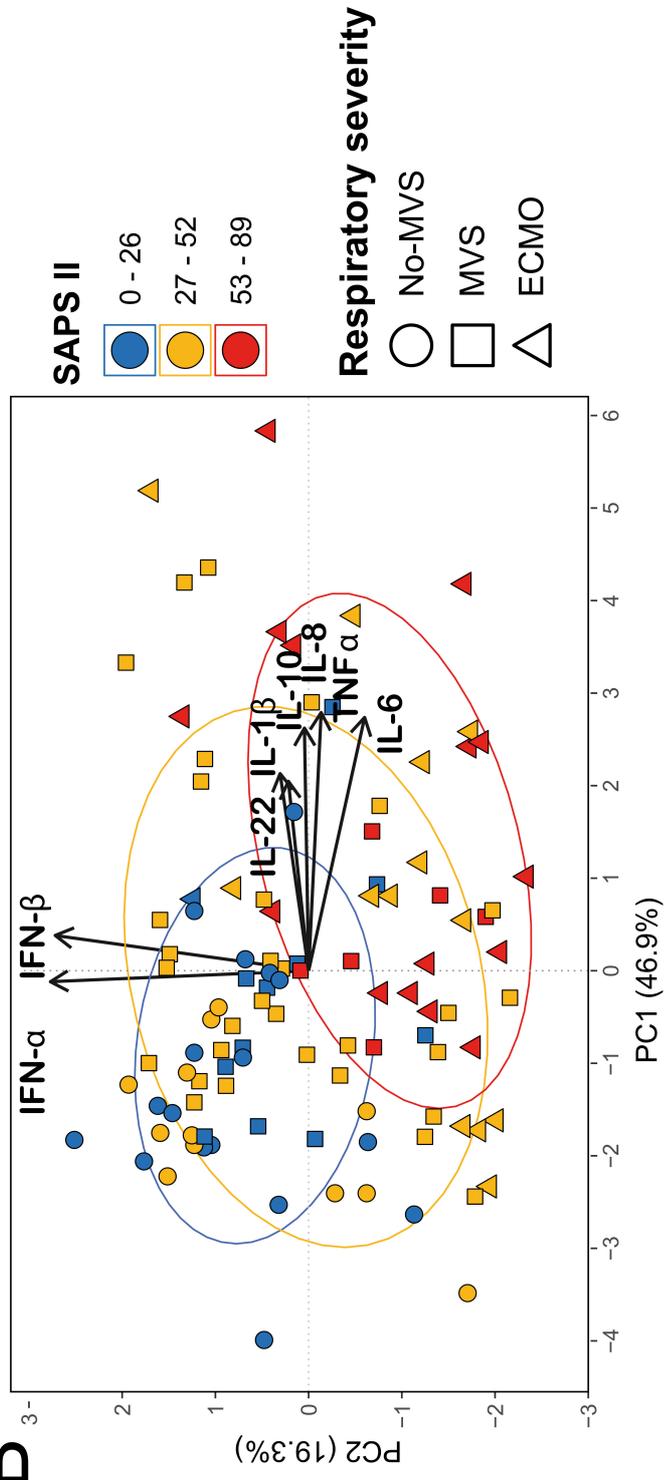
No-MVS (**A**), MVS* (**B**) and ECMO (**C**), respectively, in both validation and initial cohorts together. Ellipses represent the 68% confidence interval of patient distribution in each group. Levels of the cytokine combination functions identified in the analysis of the initial cohort for each group: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

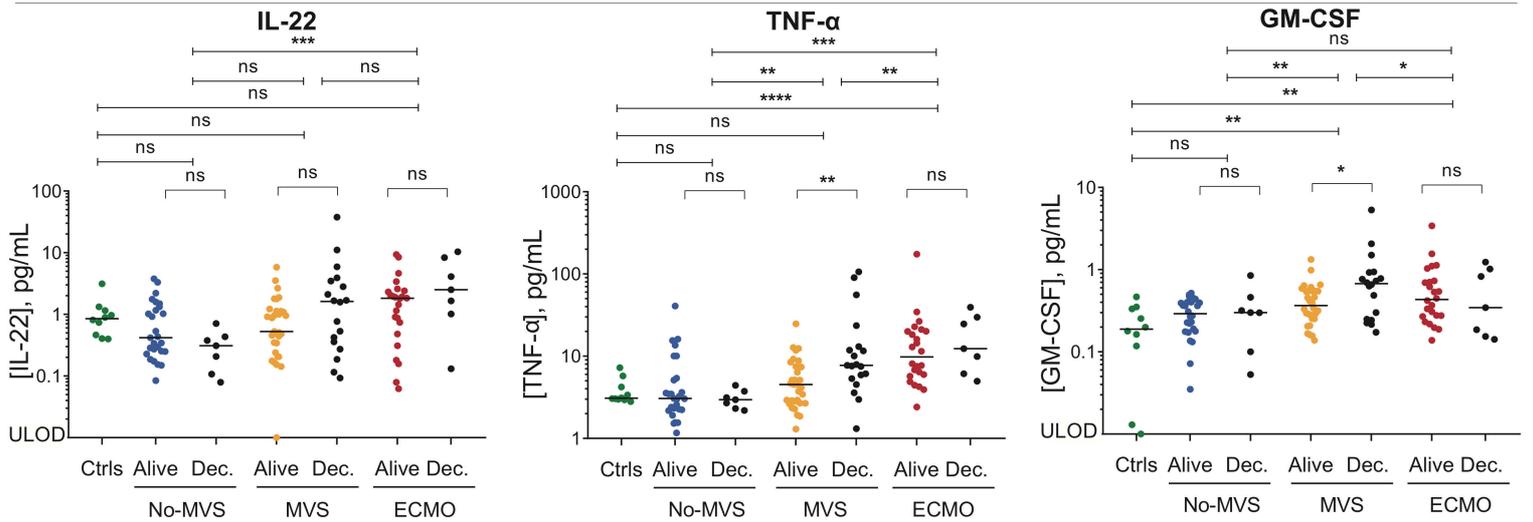
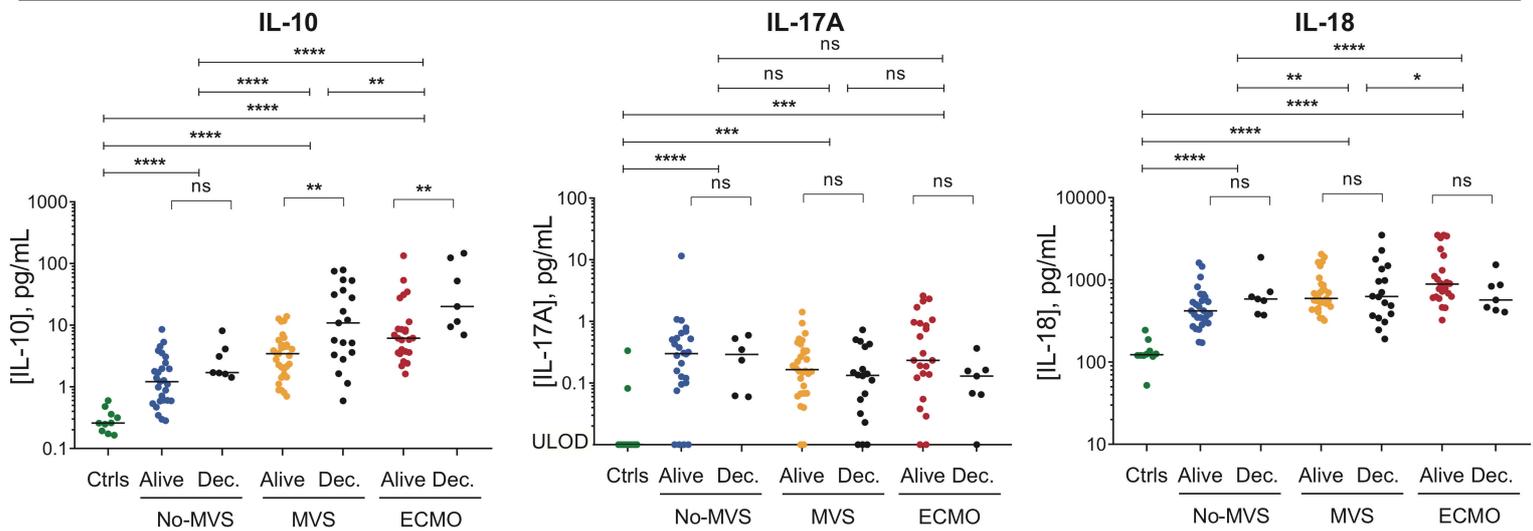
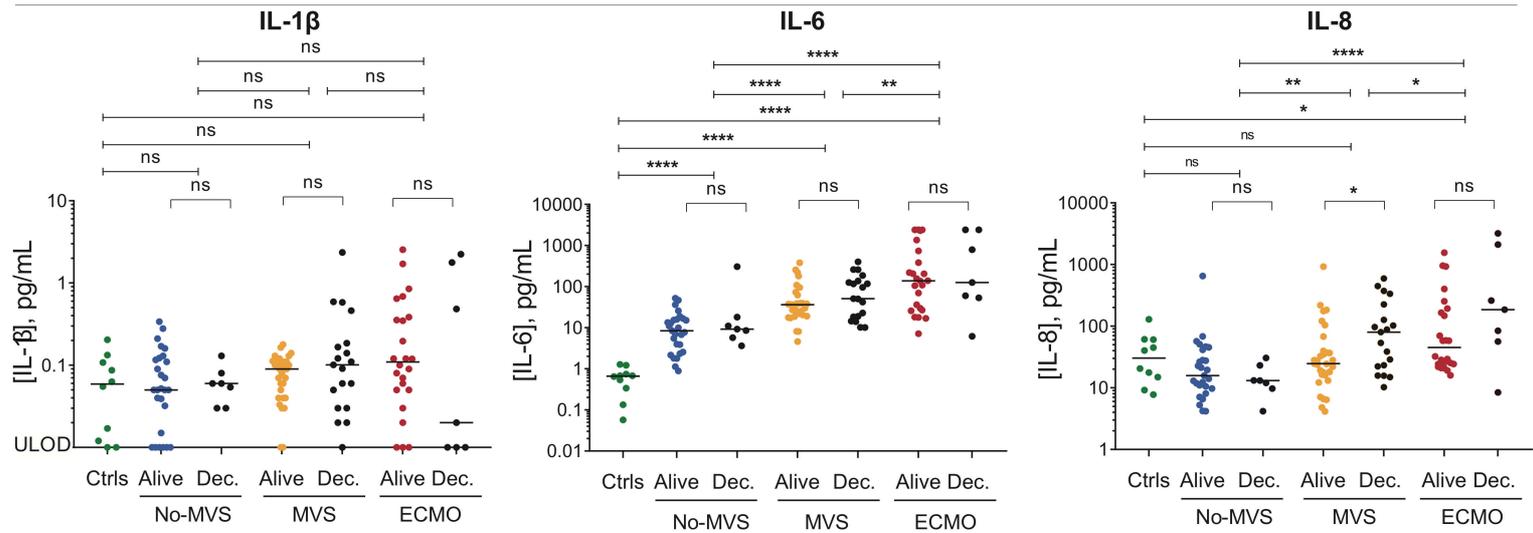
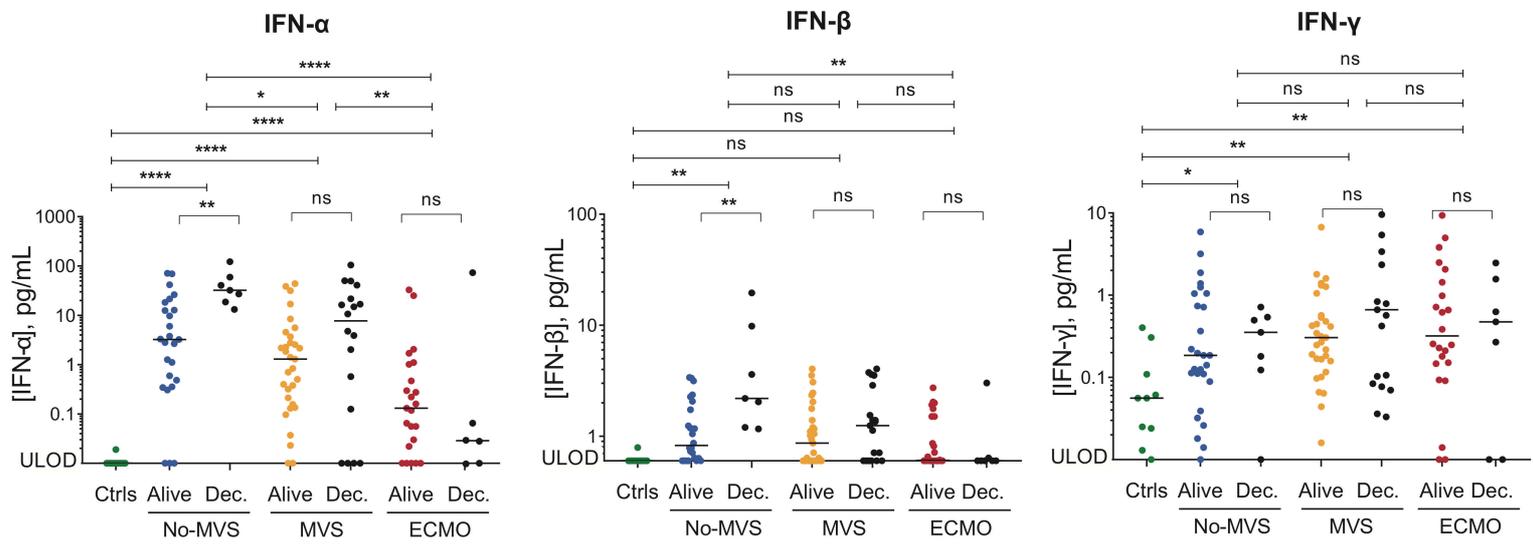
MVS*: MVS with SAPS-II ≥ 35 (median of the MVS patients). Dec.: deceased.

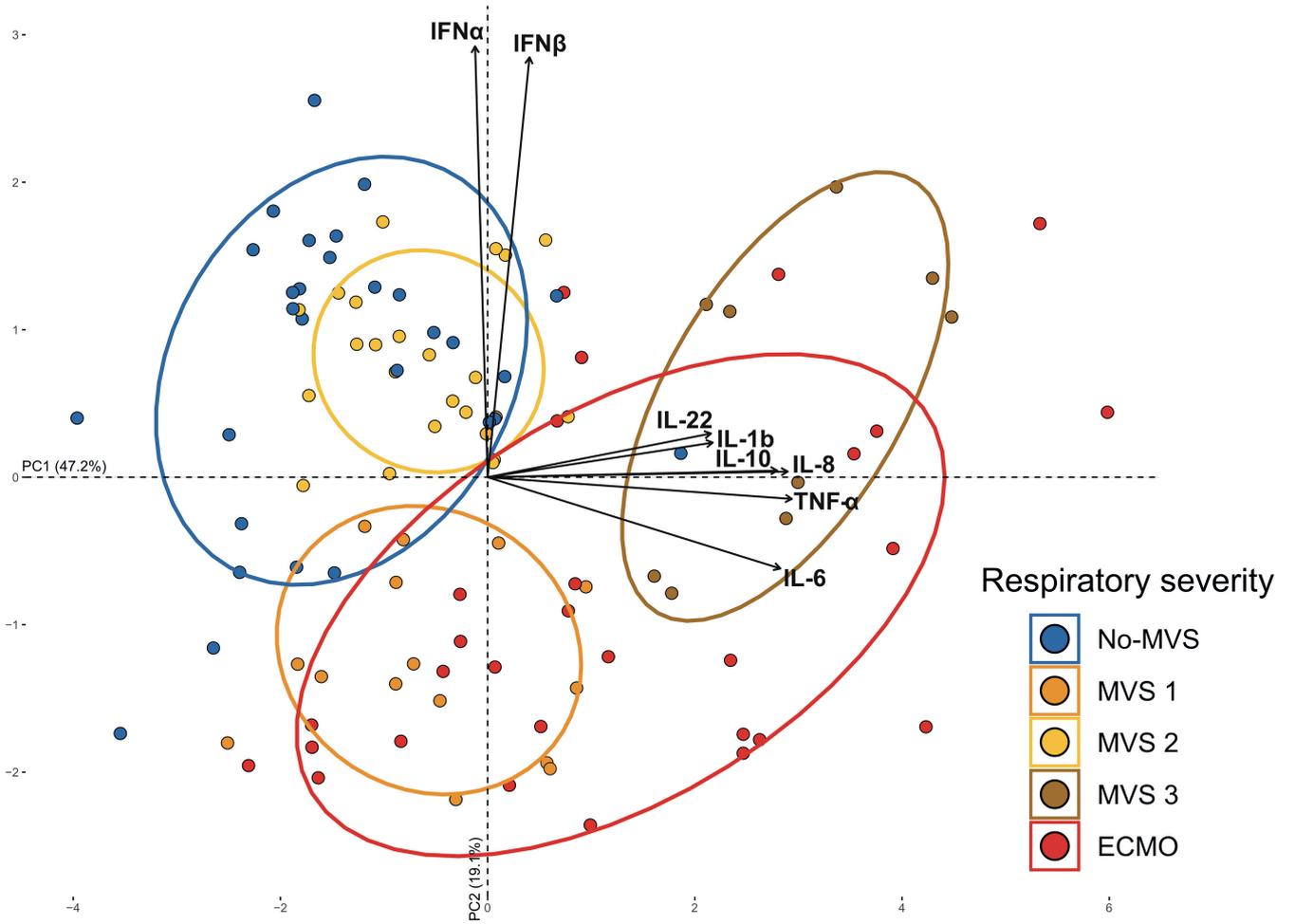
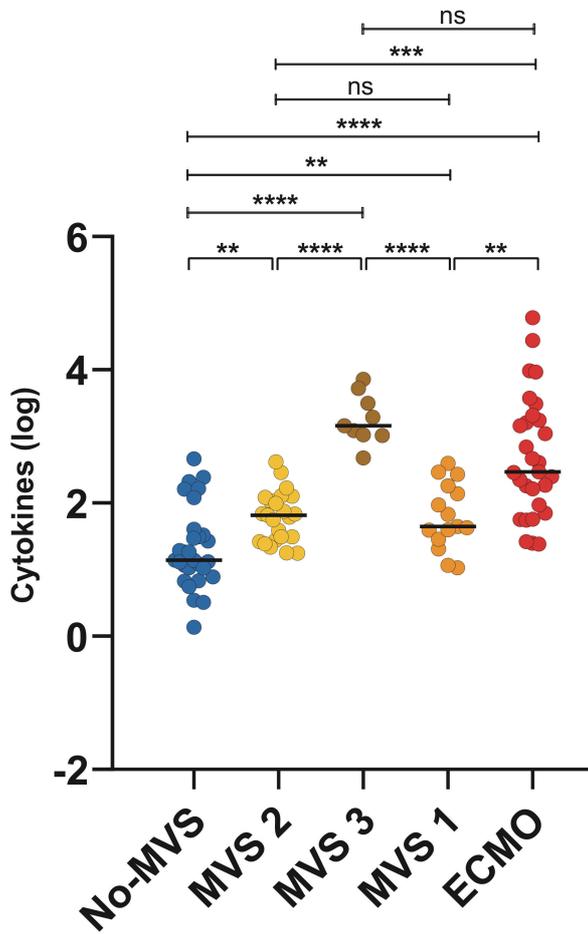
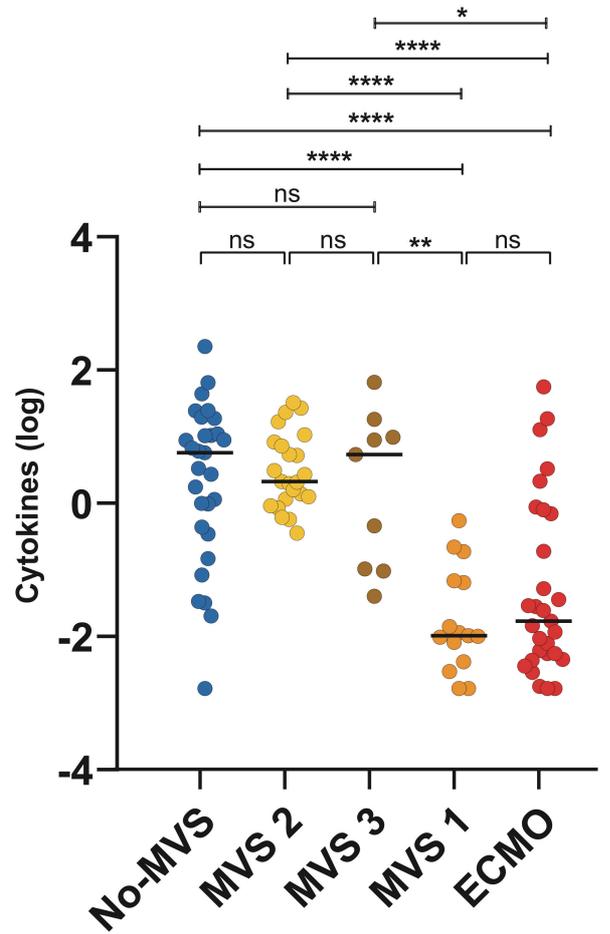
ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

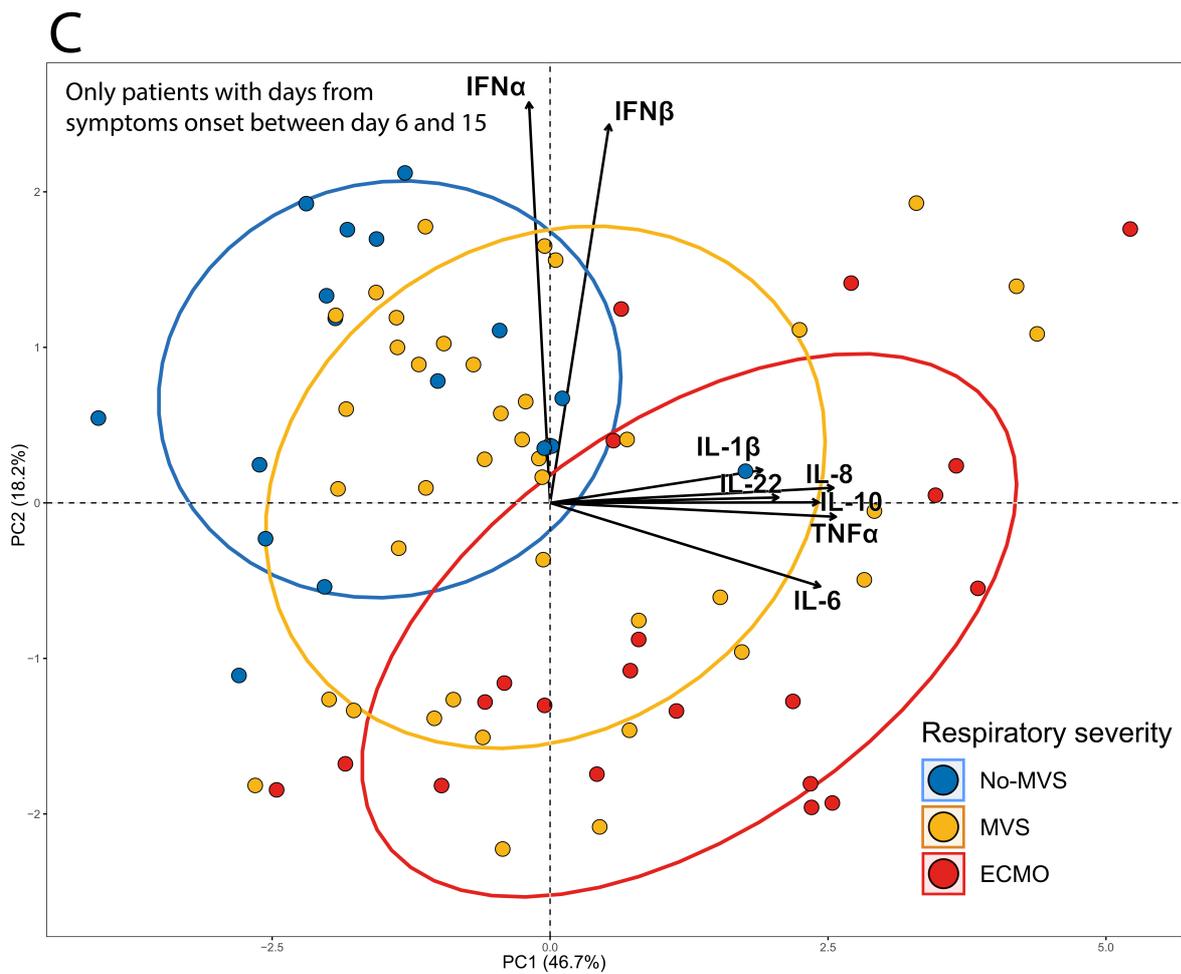
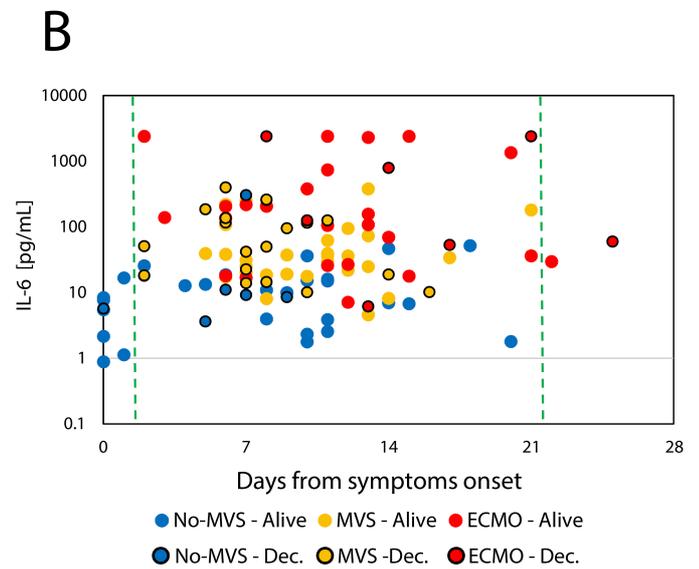
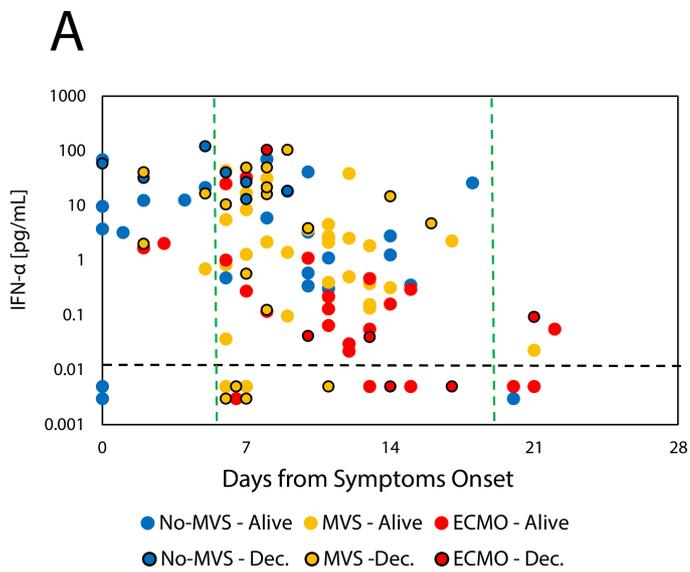
Figure E7: Receiver operating characteristic (ROC) curves of mortality outcome comparing all cytokine combination functions, SAPS and age. ROC curves for $f_{\text{No-MVS}}$, f_{MVS} , f_{ECMO} and $f_{\text{CC_INFLAM}}$, as well as SAPS and age are plotted for their predictive value of mortality in 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 $f_{\text{CC_IFN}}$ is equal to that of $f_{\text{No-MVS}}$.

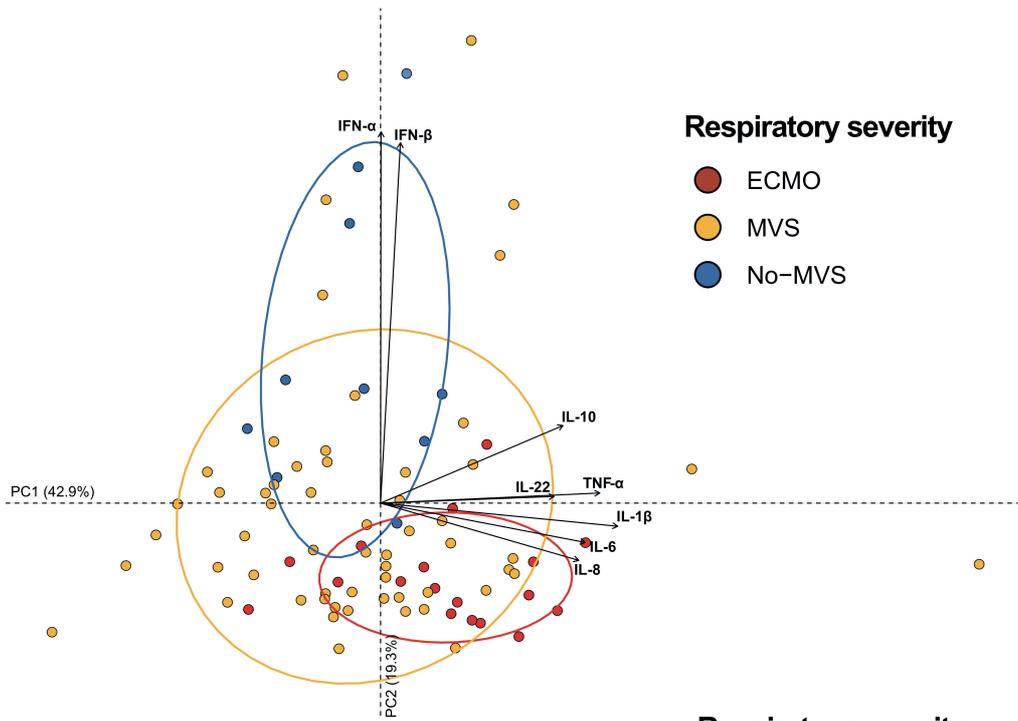
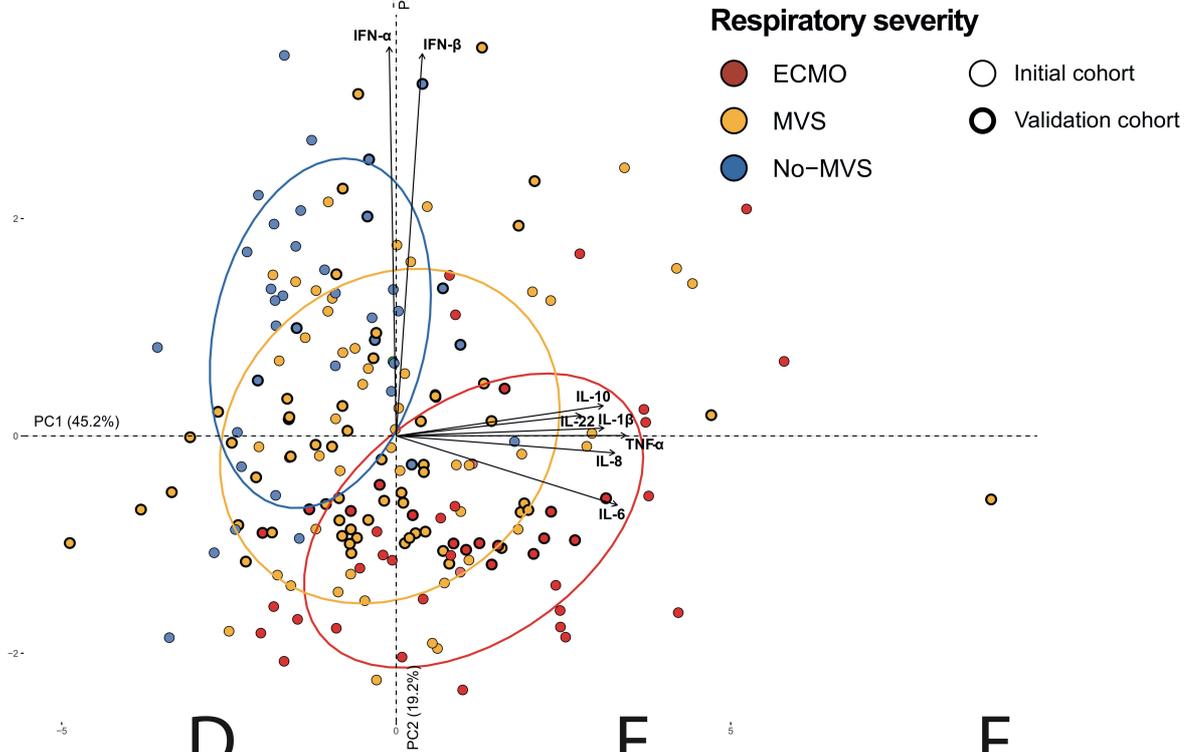
Figure E8: Receiver operating characteristic (ROC) curves of mortality outcome comparing the corresponding cytokine combination function per severity group with all individual cytokine levels. ROC curves for $f_{\text{No-MVS}}$ in No-MVS patients (**A**), f_{MVS} in MVS (SAPS ≥ 35) patients (**B**) and 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.

A**B**

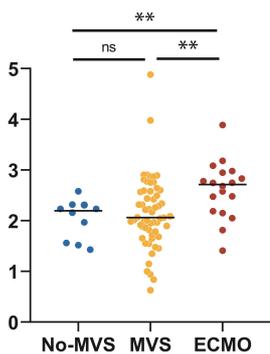


A**B****fcc-INFLAM(IL-6,IL-8,TNF- α ,IL-10)****C****fcc-IFN(IFN- α ,IFN- β)**

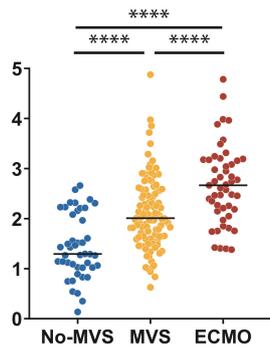


A**B****C**

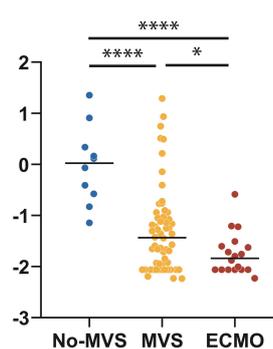
f_{CC}-INFLAM(IL-6, IL-8, TNF- α , IL-10)
Validation cohort

**D**

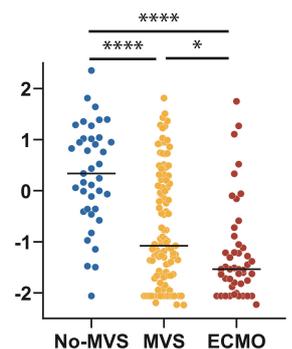
f_{CC}-INFLAM(IL-6, IL-8, TNF- α , IL-10)
All patients

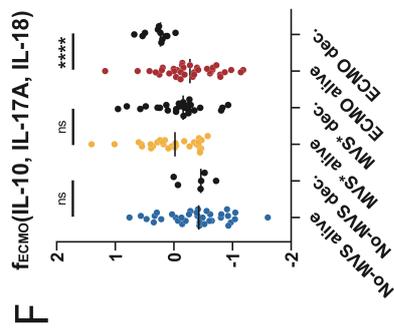
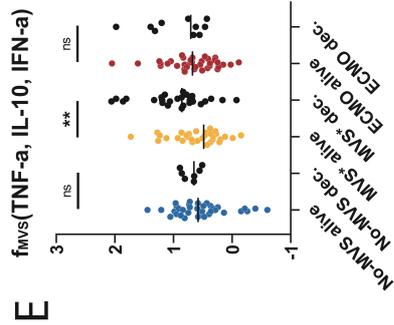
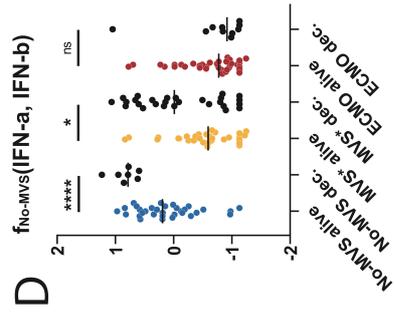
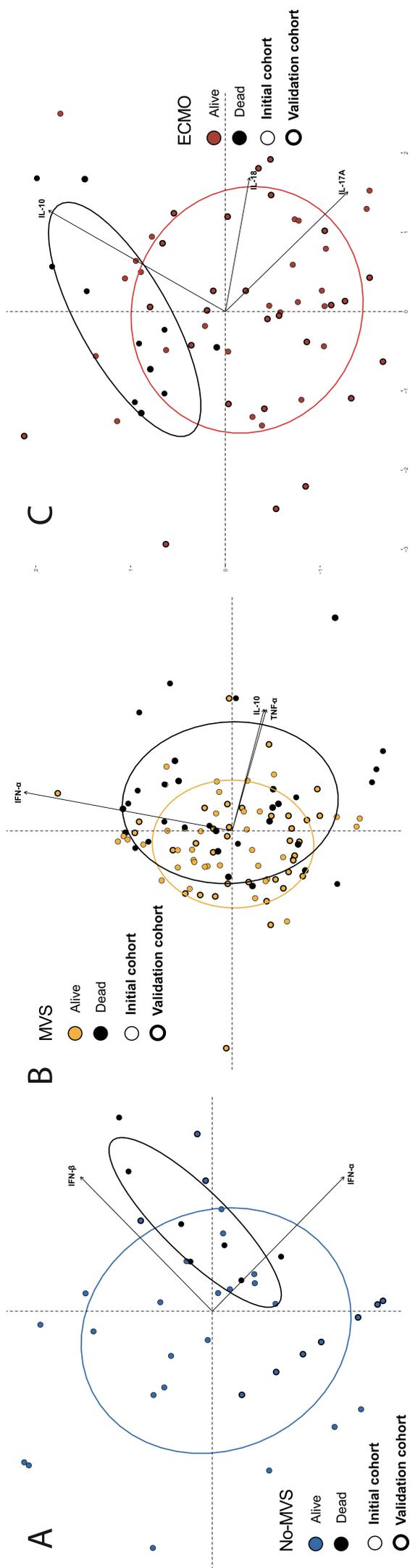
**E**

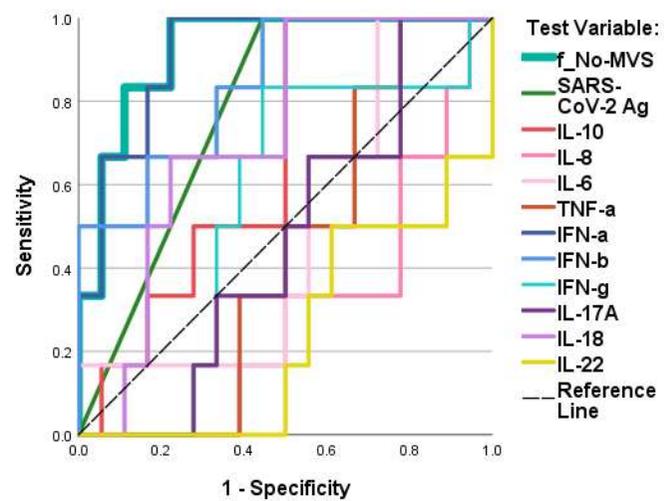
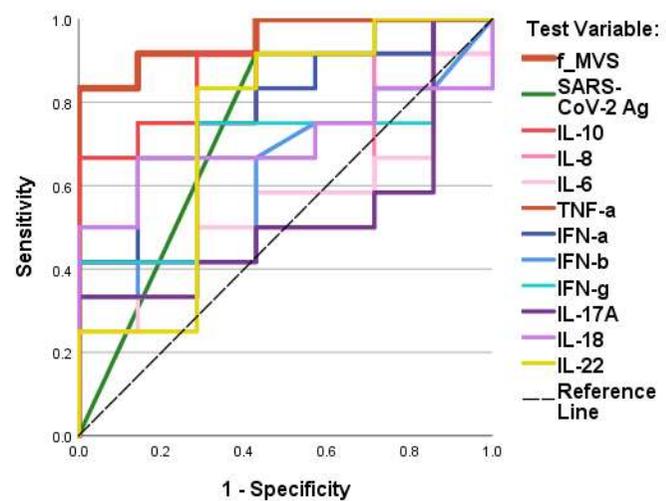
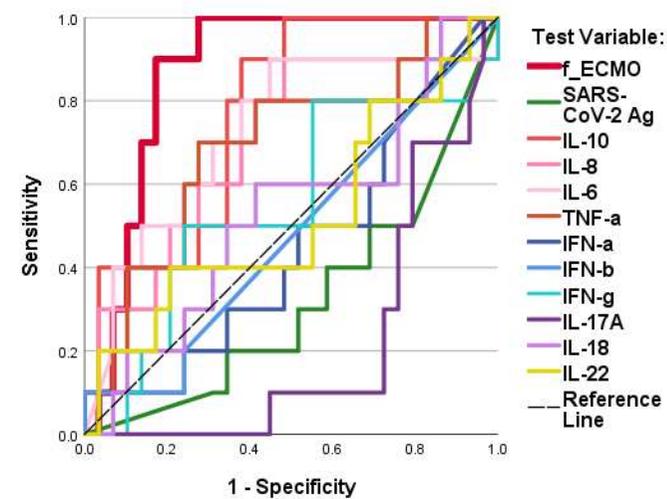
f_{CC}-IFN_I(IFN- α , IFN- β)
Validation cohort

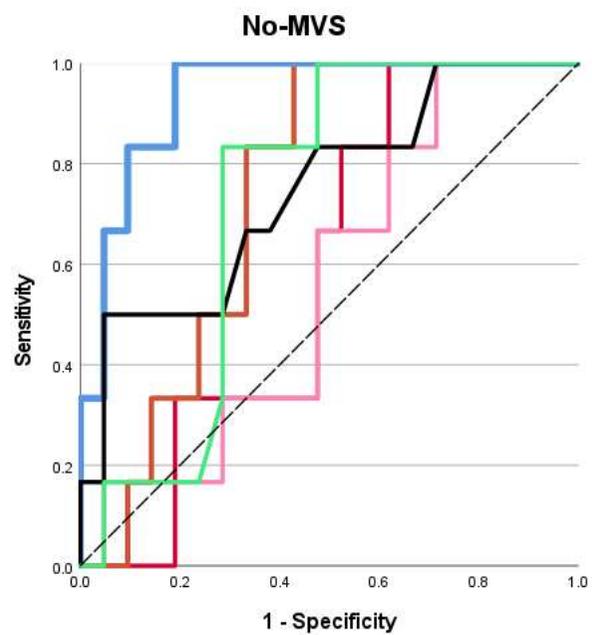
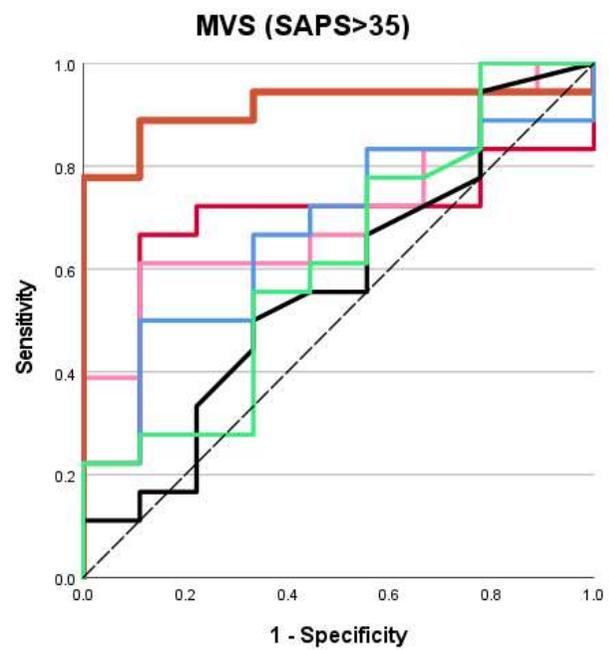
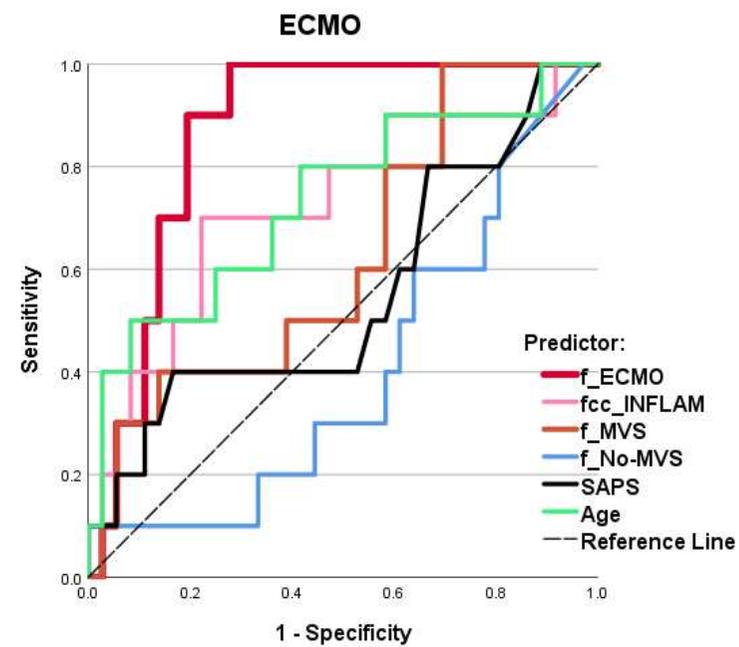
**F**

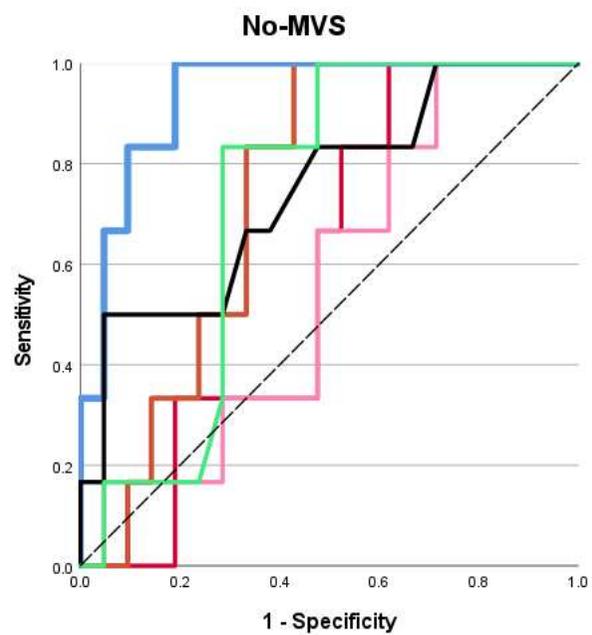
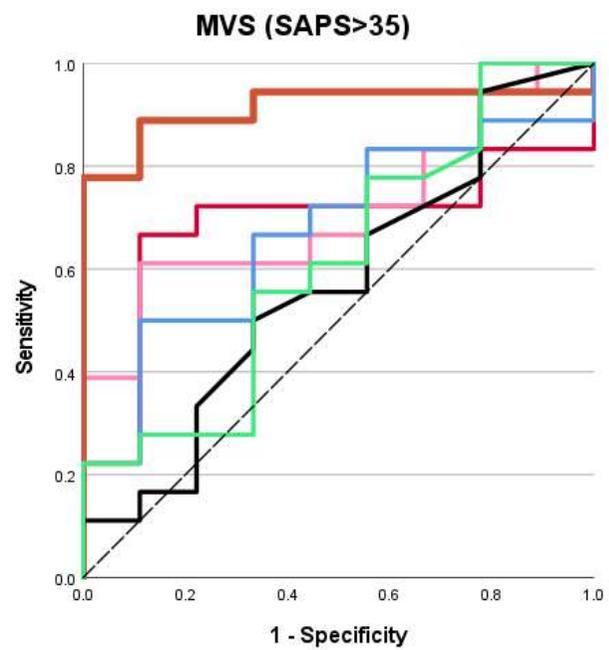
f_{CC}-IFN_I(IFN- α , IFN- β)
All patients





A**No-MVS****B****MVS (SAPS>35)****C****ECMO**

A**B****C**

A**B****C**