ORCHESTRA

Connecting European Cohorts to Increase Common and Effective Response to SARS-CoV-2 Pandemic

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Report on cytokine chemokine analysis in COVID-19 patients (including long-term sequelae)

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Executive summary

WP and deliverable context

The present report is part of ORCHESTRA project, a three-year international research project aimed at tackling the coronavirus pandemic. ORCHESTRA provides an innovative approach to learn from the pandemic SARS-CoV-2 crisis, derive recommendations to further management of COVID-19 and be prepared for the possible future pandemic waves. The ORCHESTRA project aims at delivering sound scientific evidence for the prevention and treatment of the infections caused by SARS-CoV-2 assessing epidemiological, clinical, microbiological, and genotypic aspects of population, environment, and socio-economic features. The project builds upon existing, and new largescale population cohorts in Europe (France, Germany, Spain, Italy, Belgium, Romania, Netherlands, Luxemburg, and Slovakia) and non-European countries (India, Perú, Ecuador, Colombia, Venezuela, Argentina, Brazil, Congo and Gabon) including SARS-CoV-2 infected and non-infected individuals of all ages and conditions. The primary aim of ORCHESTRA is the creation of a new pan-European cohort applying homogenous protocols for data collection, data sharing, sampling, and follow-up, which can rapidly advance the knowledge on the control and management of the COVID-19. Within ORCHESTRA project, the Work Package 6 (WP6) aims at providing innovative laboratory capabilities combining serology, immunology, viral and human genomes, microbiota and epigenetic analysis. It aims describing markers and physiopathology of various COVID-19 outcomes including severe cases, long COVID and vaccine efficiency across various patients' populations gathered within ORCHESTRA cohorts.

The objectives of the WP6 are distributed in two parts: a retrospective part on frozen samples obtained during 2020 and (2) a prospective part starting in 2021. The goal of the cytokinome analysis (Task 6.5) is to identify cytokine markers predicting disease severity, mortality, breakthrough infections and long-term sequelae in vaccinated and non-vaccinated COVID-19 patients with varying degrees of disease severity, SARS-CoV-2 positive non-symptomatic individuals and vaccinated individuals.

Content of the document

The present report describes the analysis of cytokines, chemokines and growth factors (CCG's) in two patient populations, with two different aims.

The first aim and patient population consist of 660 samples from 268 patients included from retrospective cohorts covering the years 2020 and 2021. These patients were followed-up for 12 months after hospitalization during primary disease.

The aim is to use CCG profiles in combination with clinical and demographic data:

1. To predict the risk of ARDS development at the time of hospitalization
2. To predict the chance of survival at the time of ARDS diagnosis
3. To predict the risk of long COVID development at the time of hospital discharge
4. To identify patients with high chance of having ARDS or long COVID at the time of diagnosis

The second aim and patient population consist of 402 samples from 134 patients in prospective cohorts that received monoclonal antibody therapy (mAb). We hypothesized that a host environment that is (a) less hostile to the virus and (b) facilitates tissue repair would together allow boosted cell infection cycles for rapid viral evolution under mAb pressure. To address this hypothesis, we studied 40 blood cytokines, chemokines, and growth factors as part of circulating immune-related biomarkers (CIBs) involved in either COVID-19 pathogenesis and/or wound healing. CIBs were measured at the following timepoints: (i) D0, just prior to mAb infusion; (ii) D2, 2 ± 1 days after mAb infusion on D0; (iii) D7, 7 ± 2 days after mAb infusion on D0.

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Core content

Description of the cohorts and selected samples

The numbers of included samples per cohort is depicted in the following **Table 1:**

Cohort name	Country	Type of patients	Samples included	Detection method
FrenchCOVID	France	Hospitalized	660	Meso Scale discovery assay
UNIVR monoclonal study Bamlanivimab; bamlanivimab/etesevimab; casirivimab/imdevimab; sotrovimab; tixagevimab/cilgavimab	Italy	Ambient	402	Meso Scale discovery assay

Cytokine dynamics in hospitalized patients with COVID-19

Cytokine, chemokine and growth factor (CCG) measurements in serum

All samples of interest collected before 30/10/2021 were analyzed. These samples originated from INSERM (Hôpital Bichat Claude Bernard). Forty CCGs were measured in serum samples using U-plex and V-plex panels from Meso Scale Discovery (MSD, MD, USA), according to the manufacturer instructions. The following 40 CCGs were measured: C-reactive protein (CRP), Fractalkine, Macrophage colony-stimulating factor (M-CSF), GM-CSF, Interferon γ (IFN-γ), Interleukin (IL)-4, IL-6, IL-8, IL-9, IL-10, IL-17A, IL-17F, IL-18, IL-33, IFN-γ induced protein 10 (IP-10), Monocyte chemoattractant protein (MCP)-3, Serum amyloid A (SAA), soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), Tumour necrosis factor α (TNF-α), Vascular endothelial growth factor (VEGF)-A, TSLP, total and active transforming growth factor (TGF)-β1. In addition, the following CCGs were measured in a subset of samples: B cell-attracting chemokine CXCL13 (BCA-1/BLC), basic fibroblast growth factor (bFGF), vascular endothelial growth factor receptor 1 (Flt-1), hepatocyte growth factor (HGF), IFN-a2a, IFN-beta, IL-1Ra, IL-2, IL-7, growth-regulated oncogene alpha (GRO-α), MCP-1, macrophage inflammatory protein (MIP)-1α, placental growth factor (PlGF), angiopoietin receptor 1 (Tie-2), VEGF-C, VEGF-D. Measurements were performed in randomized batches. Briefly, 96-well plates of the U-plex panels were coated with a capturing antibody linked to a linker for one hour. The vascular injury panel (K15198D) was washed before use. All plates were then washed three times with PBS-Tween (0.05%). Samples were incubated for one hour (except for the angiogenesis and the vascular injury panels, where two hours of incubation were performed), after which the plates were washed three times again. Detection antibody with a sulfo-tag was added and after another one-hour incubation step (two hours for the angiogenesis panel) plates were washed and read with MSD reading buffer on the QuickPlex SQ 120 (MSD).

All individuals were hospitalized with laboratory confirmed COVID-19. Serum samples were collected daily during hospitalization, at hospital discharge and 3, 6 and 12 months after discharge together with a clinical evaluation. In total, we received 1327 serum samples from 443 patients. For this deliverable, patients were selected based on the following criteria: All ARDS patients who died, and ARDS and non-ARDS patients with samples available for at least 2 of the following timepoints: baseline sample within 3 days of hospital admission without ARDS development, peak ARDS sample within 3 days after ARDS diagnosis in case of ARDS development or the sample during the middle of the hospital stay in case no ARDS developed, discharge day sample, 3, 6 or 12 months after initial admission. In addition, because of low availably of discharge samples, we also selected all patients who had an available discharge

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sample (**Figure 1**). Patients who did not develop ARDS (*n*=161) were randomly enriched to match those who developed ARDS (*n*= 107) 1:1.5.

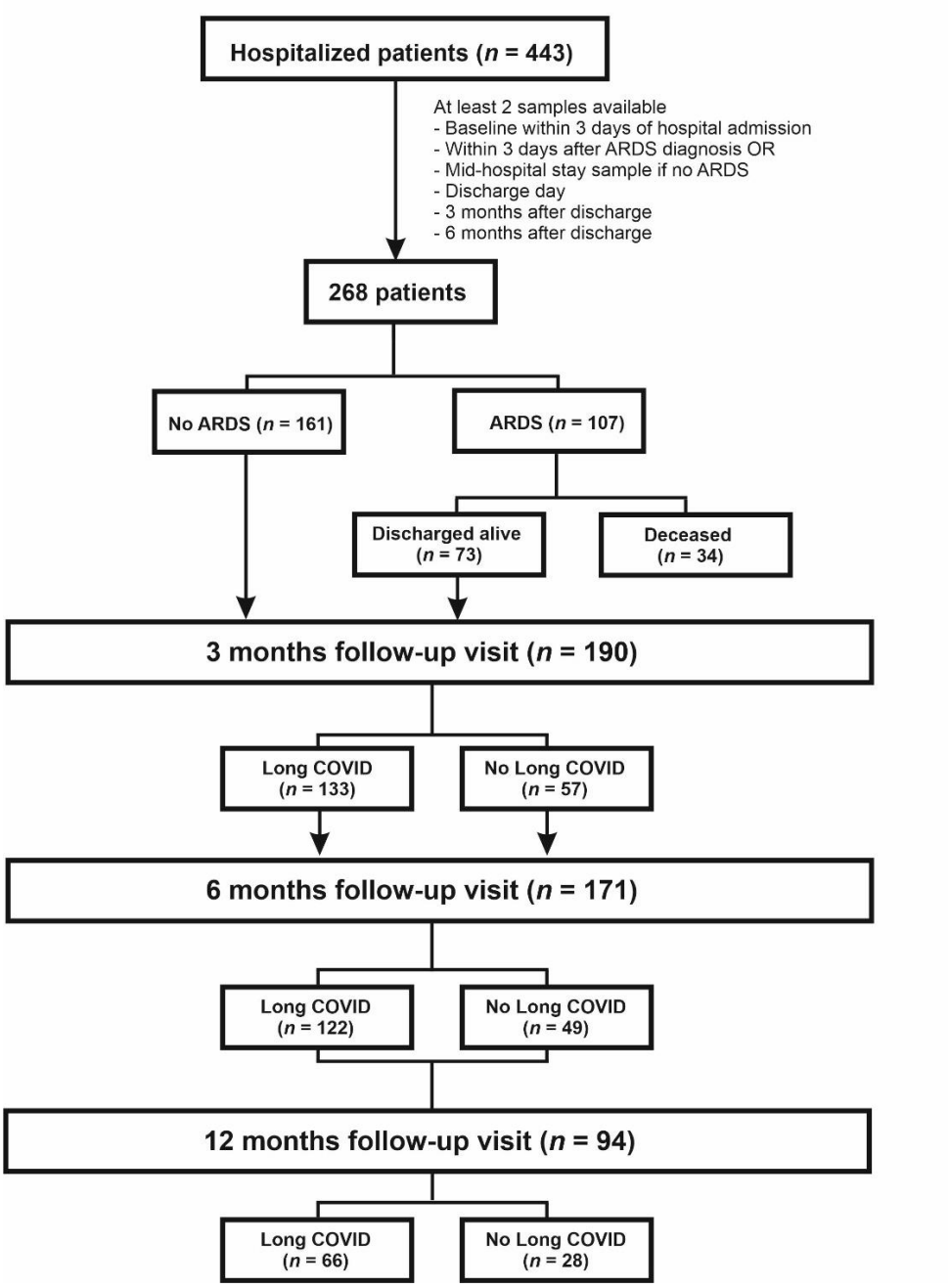

Figure 1. Patient selection and sample flow. Initially 443 hospitalized patients with laboratory confirmed COVID-19 were recruited, and patients with at least 2 samples available were further selected for downstream analysis.

Statistical analysis

All data were analyzed using SPSS v28, R (version 4.1.2), Python 3 and Metaboanalyst 5.0 (https://www.metaboanalyst.ca/ accessed on 31/10/2022). Normality was tested by Shapiro-Wilk test and skewed data were log 10 transformed before analysis using the unpaired sample T-test for pairwise comparison, one-way ANOVA with post hoc Tukey for multiple comparison or Fisher's exact test for categorical data, as appropriate. Categorical variables were compared using Chi-square test. Mixed linear model analysis was used to study cytokine changes over time for 6 months in patients with and without long COVID at 6 months

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Metaboanalyst 5.0 was used for a principal component analysis (PCA), supervised partial least squares discriminant analysis (PLS-DA) and biomarker discovery using Univariate Receiver Operating Characteristic (ROC). Data was normalized by log10 transformation and auto scaling (mean-centred and divided by the standard deviation of each variable). Analytes with more than 40% missing values were excluded and other missing values were replaced by the K means nearest neighbour method (sample wise).

In addition, Random Forest classifiers (RFC) were built by the Python package Sklearn 2.0 for the following binary outcome variables (yes or no): development of ARDS, survival at discharge or at least 1 Long Covid symptom at follow-up. To account for imbalanced data, the Synthetic Minority Oversampling Technique (SMOTE by the Python package imblearn 0.8.0) was used in combination with the above-mentioned RFC method. For this model 70% of the data was the training set and 30% the test set. The models were bootstrapped 10 times and features for each model were selected based on the union set including the top 10 potential biomarkers from the univariate ROC and feature importance of RFC. Confusion matrices and Receiver Operating Characteristic (ROC) curves were drawn to calculate area under the curve (AUROC) value to verify reliability and to evaluate the performance of the constructed models.

Preliminary results

Early predictors of ARDS development

First, we aimed to compare CCG profiles within 3 days after hospital admission of patients who did (n = 44) and did not (n = 91) develop ARDS during hospitalization and to identify potential biomarkers to predict ARDS development. At this timepoint, the pro-inflammatory cytokine IL-6 ($p < 0.001$), Interferons (IFN- γ ($p = 0.022$), IFN-β ($p = 0.002$), IP-10 (($p < 0.001$) and IFN-α2a ($p = 0.007$), immunomodulatory and anti-inflammatory cytokines IL-10 ($p = 0.003$), TSLP ($p < 0.001$) and IL-9 ($p = 0.001$), chemo-attractants MCP-3 ($p < 0.001$) and Fractalkine ($p < 0.01$) and colony stimulating factors M-CSF ($p = 0.001$) and GM-CSF ($p < 0.001$) were significantly upregulated whereas immunomodulatory cytokine IL-10 ($p < 0.01$) was significantly downregulated in patients who developed ARDS later during hospitalization.

The random forest model was used to rank the importance of the CCGs measured at the time of hospital admission based on their ability to discriminate between patients who developed ARDS or not. IP-10 was ranked as the most important discriminator, forming the top 10 with MCP-3, IFN-γ, TSLP, IL-6, IL-9, SAA, IL-10, CRP and sVCAM-1. In addition, classical univariate ROC curve analysis was performed to identify individual cytokines with a discriminative power of 70% or more, including IP-10, IL-6, MCP-3, TSLP, IL-10, IFN-γ, Fractalkine and IL-9 (**Table 2**). A union set of the top 10 cytokines identified by the random forest model and having an individual discriminative power of >70% in the univariate ROC curve analysis were further combined in a multivariate ROC analysis combining 3 cytokines to increase predictive power. The highest predictive power was obtained by using a combination of IP-10, MCP-3 and IFN-γ (mean AUROC 0.891 ± 0.048).

Table 2: ROC curve analysis of CCG biomarkers predicting the development of ARDS at the time of hospitalization. Top 10 AUROC is shown.

Name	AUROC	T-tests	log2 fold Change ARDS/NoARDS
IP-10	0.809	< 0.001	1.37
MCP-3	0.800	< 0.001	1.04
IL-6	0.759	< 0.001	-0.37
TSLP	0.744	< 0.001	0.99
IL-10	0.697	0.003	-1.74

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CRP	0.679	0.499	0.48
Fractalkine	0.677	0.002	0.57
VEGF-C	0.670	0.195	-0.15
IL-9	0.666	0.001	0.35
SAA	0.656	0.661	0.41

Cytokines distinguishing ongoing ARDS from no ARDS during the middle of the hospital stay

Here we compared CCG profiles of ARDS patients within 3 days after ARDS diagnosis and the mid-hospitalization timepoint for patients who did not develop ARDS. At this timepoint, pro-inflammatory cytokines IL-6 ($p < 0.001$), TNF-α ($p = 0.042$), IL-8 ($p < 0.001$) and CRP ($p = 0.001$), Interferon-response proteins (IP-10 ($p < 0.001$), IL-18 ($p = 0.001$) and IFN-α2a ($p = 0.042$)),immunomodulatory cytokines IL-10 ($p < 0.001$), IL-4 ($p = 0.003$), TSLP ($p < 0.001$) and IL-17A ($p = 0.003$), chemokines MCP-3 ($p < 0.001$) and Fractalkine ($p = 0.002$), colony stimulating factors M-CSF ($p < 0.001$) and GM-SCF ($p < 0.001$) and adhesion molecules sICAM-1 ($p < 0.001$) and sVCAM-1 ($p < 0.001$) were significantly upregulated in patients with ARDS compared to the timepoint in the middle of the hospital stay of patients who never developed ARDS.

Similar to predicting ARDS above, we next investigated whether cytokine profiles could be used to distinguish patients with ongoing ARDS from those without ARDS. The top 10 discriminative CCGs of the RFC analysis consists of GM-CSF, MCP-3, IL-10, IL-8, TSLP, IL-17-F, sVCAM-1, CRP, IL-6 and IP-10. The top 10 classifiers by univariate ROC are GM-CSF, MCP-3, TSLP, IL-6, sVCAM-1, IL-10, sICAM-1, CRP, IP-10 and M-CSF (**Table 3**). The highest predictive value was obtained by combining MCP-3, GM-CSF and TSLP, with a mean AUROC of 0.951 ± 0.060.

Table 3: ROC curve analysis of CCG biomarkers distinguishing ARDS from no ARDS at the middle of the hospital stay. Top 10 AUROC is shown.

Name	**AUC**	**T-tests**	**log2 fold Change ARDS/NoARDS**
GM-CSF	0.880	< 0.001	4.27
MCP-3	0.878	< 0.001	2.79
TSLP	0.840	< 0.001	2.05
IL-6	0.819	< 0.001	4.32
sVCAM-1	0.807	0.009	0.83
IL-10	0.805	< 0.001	2.27
sICAM-1	0.791	0.003	0.75
CRP	0.779	0.001	1.02
IP-10	0.761	< 0.001	1.53
M-CSF	0.743	< 0.001	1.22

Predictors of survival during ARDS

Next, we aimed to investigate the CCG profiles before and during ARDS of patients who were discharged alive ($n = 67$) and died during hospitalization ($n = 43$) to identify potential biomarkers predicting survival during ARDS. Here, the pro-inflammatory cytokines IL-6 ($p < 0.001$), TNF-α ($p < 0.01$), IL-8 ($p < 0.01$), CRP ($p < 0.001$) and SAA ($p < 0.01$), Interferon-response proteins IFN-γ ($p < 0.01$), IL-18 ($p < 0.001$) and IFN-α2a ($p = 0.001$), Immunomodulatory cytokines IL-10 (($p < 0.01$), IL-4 ($p < 0.05$), TSLP ($p < 0.001$) and IL-17A ($p < 0.001$), chemokines MCP-3 ($p < 0.001$) and Fractalkine ($p < 0.001$), colony stimulating factors M-CSF ($p < 0.001$) and GM-CSF ($p < 0.001$), growth factor VEGF-C

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($p < 0.05$), growth factor receptors Flt-1 ($p < 0.05$) and Tie-2 ($p < 0.01$) and adhesion molecules sICAM-1 ($p = 0.001$) and sVCAM-1 ($p = 0.001$) were significantly upregulated in patients who died after ARDS development whereas IL-17F was significantly downregulated ($p = 0.001$).

Random forest classification ranked IP-10 as the most important discriminator between patients who died and survived during ARDS, followed by M-CSF, IL-6, MCP-3, CRP, IL-18, TSLP, IFN-γ, GM-CSF and IL-17A forming the top 10. The top 10 CCGs identified by univariate ROC curve analysis ranked M-CSF as having the highest discriminative power (AUC: 0.82) followed by MCP-3, IL-6, CRP, sICAM-1, TSLP, IP-10, GM-CSF, sVCAM-1 and IL-17A (**Table 4**). Combinations of 3 cytokines from the union set of both classification methods, preferably different from the combination used to predict ARDS development, were selected for multivariate ROC analysis. The highest predictive power was obtained by combining CRP, TSLP and M-CSF (mean AUROC 0.920 ± 0.041).

Table 4: ROC curve analysis of CCG biomarkers distinguishing ARDS patients that were discharged alive or died during ARDS. Top 10 AUROC is shown.

Name	AUC	T-tests	log2 fold Change Death/Alive
M-CSF	0.887	< 0.001	1.69
sICAM-1	0.866	0.005	0.72
TSLP	0.851	< 0.001	1.77
IL-6	0.821	< 0.001	1.28
CRP	0.819	0.008	0.72
Fractalkine	0.799	< 0.001	0.80
IP-10	0.793	< 0.001	1.09
MCP-3	0.790	< 0.001	1.06
sVCAM-1	0.765	0.039	0.50
IL-17F	0.759	0.022	-0.70

Early predictors of long COVID at 3 months

We investigated whether the CCG profiles at the time of hospital discharge can be used to predict long COVID symptoms after 3 months. We measured the CCG profile in 32 patients with and 15 patients without symptoms at month 3. Here, only Fractalkine ($p = 0.032$) and VEG-F D ($p = 0.041$) were significantly downregulated in patients who had symptoms at month 3.

Random forest classification ranked VEGF-D, IL-2, GM-CSF, IFN- α2a, sICAM-1, IL-17F, VEGF-A, Flt-1, Fractalkine and PlGF as the top 10 important discriminators between patients who had symptoms at month 3 and who did not. The top 10 CCGs identified by univariate ROC curve analysis were Fractalkine, VEGF-D, IFN- α2a, GM-CSF, bFGF, Flt-1, IL-17F, M-CSF, MCP-1 and IL-1Rα (**Table 5**). The best discrimination based on multivariate ROC was IL-17F, IL-33 and M-CSF (mean AUROC 0.901 ± 0.163).

Table 5: ROC curve analysis of CCG biomarkers at hospital discharge predicting symptoms at M3. Top 10 AUROC is shown.

Name	AUC	T-tests	log2 fold Change LTS/No LTS
Fractalkine	0.669	0.032	-0.58
VEGF-D	0.635	0.041	-0.24
IFN-a2a	0.633	0.273	0.34
GM-CSF	0.625	0.097	-0.07

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bFGF	0.625	0.102	0.10
Flt-1	0.617	0.631	-0.05
IL-17F	0.608	0.116	0.32
M-CSF	0.606	0.311	-0.58
MCP-1	0.594	0.926	0.05
IL-1Ra	0.593	0.583	0.22

Markers for long COVID at 3 months, 6 months, and 12 months

We also compared the CCG profiles at 3 and 6 months after primary disease, to explore whether these could be used to distinguish people with and without symptoms at these timepoints. At 3 months, we analyzed 99 samples from people with and 39 samples from people without symptoms. At 6 months, these were 66 and 25 samples and at 12 months 39 and 18 samples, respectively.

At 3 months, the anti-inflammatory cytokines IL-33 ($p < 0.001$) and IL-2 ($p = 0.023$) and growth factor receptor Tie-2 ($p = 0.007$) were significantly downregulated in patients with symptoms. At 6 months, acute phase proteins CRP ($p = 0.010$) and SAA ($p = 0.011$) and adhesion molecules sICAM-1 ($p = 0.019$) and sVCAM-1 ($p = 0.040$) were significantly upregulated, whereas anti-inflammatory cytokine IL-33 ($p = 0.034$) was significantly downregulated in patients with symptoms. Last, at 12 months anti-inflammatory cytokine IL-17A ($p = 0.015$) was significantly downregulated in patients with symptoms.

At 3 months, random forest classification ranked sVCAM-1, Tie-2, IL-33, MCP-3, IL-18, sICAM-1, SAA, TSLP. MIP-1α and IL-2 as the top 10 important discriminators between patients who had symptoms and who did not. The top 10 CCGs identified by univariate ROC curve analysis were IL-33, SAA, CRP, IL-2, PlGF, IFN-α2a, IFN-β, Tie-2 and total TGF-β (**Table 6**). The best discrimination based on multivariate ROC was IL-17F, IL-33 and VEGF (mean AUROC 0.942 ± 0.040).

Table 6: ROC curve analysis of CCG biomarkers distinguishing patients with and without symptoms at 3 months. Top 10 AUROC is shown. LTS: Long term Sequelae.

Name	AUC	T-tests	log2 fold Change LTS/No LTS
IL-33	0.641	< 0.001	-1.59
SAA	0.603	0.104	1.40
CRP	0.599	0.054	1.44
IL-2	0.596	0.023	-0.65
IL-10	0.581	0.162	-0.08
PlGF	0.580	0.468	-0.10
IFN-a2a	0.578	0.189	2.92
IFN-b	0.574	0.107	-0.79
Tie-2	0.573	0.007	-0.13
TGF-b Total	0.561	0.204	0.25

At 6 months, random forest classification ranked CRP, SAA, sICAM-1, PlGF, IFN-β, M-CSF, HGF, GRO-α, VEGF-C and MIP-1α as the top 10 important discriminators between patients who had symptoms and who did not. The top 10 CCGs identified by univariate ROC curve analysis were SAA, sICAM-1, CRP, sVCAM-1, PlGF, GRO- α, IL-8, M-CSF, IL-33 and HGF (**Table 7**). The same profile as month 3 was implemented in multivariate ROC (IL-17F, IL-33 and VEGF), leading to a mean AUROC of 0.870 ± 0.073.

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Table 7: ROC curve analysis of CCG biomarkers distinguishing patients with and without symptoms at 6 months. Top 10 AUROC is shown. LTS: Long term Sequelae.

Name	AUC	T-tests	log2 fold Change LTS/No LTS
SAA	0.695	0.011	1.69
sICAM-1	0.672	0.019	0.64
CRP	0.661	0.010	1.47
sVCAM-1	0.637	0.040	0.42
PlGF	0.619	0.122	0.22
GRO-a	0.611	0.519	-0.54
IL-8	0.607	0.447	0.13
M-CSF	0.605	0.587	-0.16
IL-33	0.605	0.034	-2.30
HGF	0.586	0.738	-0.23

At 12 months, random forest classification ranked IL-17A, bFGF, TSLP, VEGF-C, Flt-1, IP-10, M-CSF, Fractalkine, IL-8, and IL-18 as the top 10 important discriminators between patients who had symptoms and who did not. The top 10 CCGs identified by univariate ROC curve analysis were IL-17A, bFGF, M-CSF, IL-4, TSLP, CRP, TNF-α, IP-10, VEGF-C and Flt-1. The best discrimination based on multivariate ROC was IL-17A, bFGF, TSLP and M-CSF (mean AUROC 0.872 ± 0.114).

Table 8: ROC curve analysis of CCG biomarkers distinguishing patients with and without symptoms at 12 months. Top 10 AUROC is shown. LTS: Long term Sequelae.

Name	AUC	T-tests	log2 fold Change LTS/No LTS
IL-17A	0.704	0.015	-3.21
bFGF	0.638	0.233	0.92
M-CSF	0.624	0.074	0.31
IL-4	0.595	0.206	-0.72
TSLP	0.591	0.891	0.75
CRP	0.585	0.485	0.28
TNF-a	0.571	0.210	0.11
IP-10	0.570	0.320	-0.13
VEGF-C	0.570	0.413	-0.06
Flt-1	0.568	0.206	0.23

Longitudinal cytokine dynamics for 6 months after primary disease

Lastly, we used mixed linear model analysis to investigate cytokine dynamics over 6 months in patients with and without symptoms at this timepoint. Here, the change in CRP ($p < 0.05$), IP-10 ($p < 0.05$), IL-18 ($p < 0.01$), GRO-α ($p < 0.05$), HGF ($p < 0.05$), BCA/BLC ($p < 0.01$), IL-7 ($p < 0.05$) and IL-1Rα ($p < 0.05$) were significantly different over time in patients with and without symptoms at 6 months (**Figure 2**), whereas IL-9 ($p < 0.05$), Active TGF-β ($p < 0.01$), and sVCAM-1 ($p < 0.05$) concentrations were significantly different at the time of hospital admission (Figure 2).

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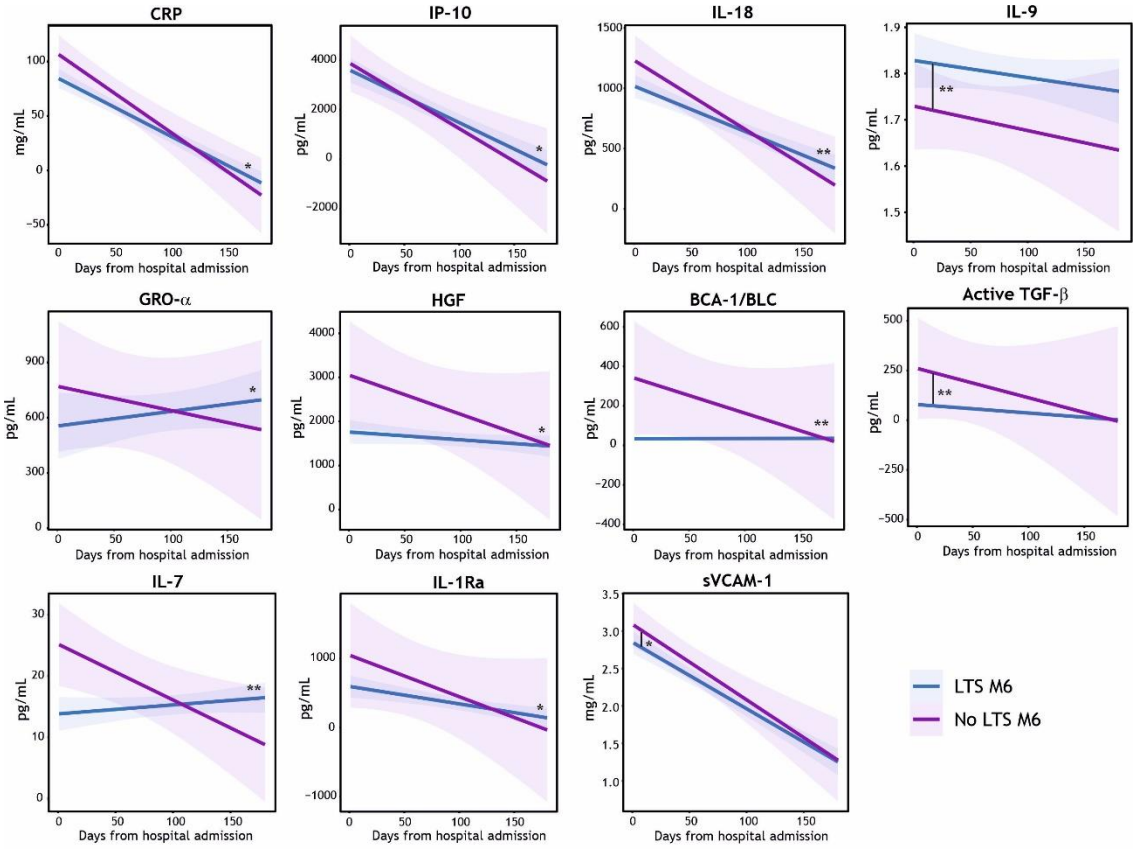

Figure 2: Mixed linear model analysis to study cytokine dynamics over time for 6 months in patients with and without long COVID symptoms. LTS: LTS: Long term Sequelae; M6: 6 months timepoint; * $p < 0.05$; ** $p < 0.01$.

Output

Part of these results have been presented as a flash ePoster at the ECCMID conference 2023.

An Hotterbeekx, Samuel Lebourgeois, Angelina Konnova, Reyene Menidjel, Sarah Tubiana, Akshita Gupta, Matilda Berkell, Cédric Laouénan, Elisa Gentilotti, Anna Gorska, Surbhi Malhotra-Kumar, Evelina Tacconelli, Jade Ghosn, Charlotte Charpentier, Samir Kumar-Singh, *Cytokine dynamics in the acute and post-acute phase of hospitalized COVID-19 patients and their link to disease severity, mortality, and Long COVID;* ECCMID Abstract 01814;

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Cytokine dynamics in COVID-19 patients who received monoclonal antibody therapy

Cytokine, chemokine and growth factor (CCG) measurements in serum

CIBs were measured in serum samples using U-plex and V-plex panels (K15198D and K15190D) on the QuickPlex SQ 120 system (Meso Scale Discovery), according to the manufacturer's instructions. The following 40 CIBs were measured for the D0, D2, and D7 time points: bFGF, CRP, cutaneous T cell–attracting chemokine (CTACK), eotaxin, erythropoietin (EPO), Flt-1, fractalkine, M-CSF, IFN-β, IFN-γ, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-2 receptor α (IL-2Rα), IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-15, IL-17A, IL-17F, IL-18, IL-22, IL-33, IFN-γ–induced protein 10 (IP-10), MCP-1, MCP-2, MCP-3, macrophage inflammatory protein 1α (MIP-1α), PlGF, SAA, soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), Tie-2, tumor necrosis factor α (TNF-α), VEGF-A, VEGF-C, and VEGF-D. A small panel of 4 select CIBs, consisting of CRP, bFGF, Tie2, and M-CSF, was additionally utilized for validating CIB profile predictive of SARS-CoV-2 mutations.

Statistical analysis

All data were statistically analyzed and visualized in Rstudio v.1.3.1073 (https://github.com/rstudio/rstudio) using R v.4.0.4 (https:// www.r-project.org/). One-way analysis of variance (ANOVA) was utilized for longitudinal and cross-sectional comparisons of CIB concentrations across treatment groups followed by pairwise 2-tailed t tests. Post hoc P-value correction was conducted using Bonferroni's multiple-comparison correction method. Throughout the statistical analyses, values below the detection range were recorded as 1/10 the lower limit of quantitation (LLQ) and values above the detection range were recorded as upper limit of quantitation (ULQ). A (corrected) P value of less than 0.05 was considered statistically significant. For the identification of the main predictors of qualitative responses (mutation/no mutation in the S RBD region [residues 319– 541]), ROC curves were constructed utilizing MetaboAnalyst (https://dev.metaboanalyst.ca/MetaboAnalyst/). Machine learning–based random forest classifiers (RFCs) were further built by the Python package sklearn v3.10 (https://www.python.org/) to independently predict development of de novo S RBD mutations in patients receiving mAb regimens. Each model was built with a training set of values consisting of 70% of the data and a test set of 30% (57). To account for imbalanced groups, the synthetic minority oversampling technique (SMOTE, Python package imblearn 0.8.0) was utilized in combination with the RCF method. The models were bootstrapped 100 times and features for each model were selected based on (a) feature importance, (b) statistics from mutation versus nonmutation, (c) individual ROC curve analysis, and (d) a Pearson correlation matrix for independence of variables. Confusion matrices and ROC curves were drawn to calculate AUROC values to verify reliability and to evaluate the performance of the constructed models. The CIB model built to predict emergence of evasive SARS-CoV-2 S RBD mutations in patients treated with mAbs in the main study population was validated both by RFC and binomial logistic regression in a patient cohort on independently generated data sets. Linear mixed models were utilized to investigate evolution of antibody titers and Th cell immunity over time between the different mAb groups.

Main results

Host immune profile as a predictor of S RBD escape mutants.

Studies have shown that proinflammatory cytokines, when uncontrolled and exaggerated, can lead to immunopathogenesis such as cytokine release syndrome disorder; however, under homeostatic conditions they are believed to play a major role in the control and resolution of SARS-CoV-2 infection. Moreover, cytokines along with growth factors are critical to fundamental homeostatic processes such as wound healing and tissue repair. We hypothesized that a host environment that is (a) less hostile to

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the virus and (b) facilitates tissue repair would together allow boosted cell infection cycles for rapid viral evolution under mAb pressure.

To address this hypothesis, we studied 40 blood cytokines, chemokines, and growth factors as part of circulating immune-related biomarkers (CIBs) involved in either COVID-19 pathogenesis and/or wound healing. Significant changes between different treatment groups occurred in the levels of 34 of 40 (85.0%) cytokines (**Figure 3**) that are also linked to infection with different SARS-CoV-2 variants. We further utilized area under the curve receiver operating characteristic (AUROC) analysis to discriminate between patients developing de novo S RBD mutations from those who did not or those who rapidly cleared the virus. AUROC for CIBs just before mAb administration identified 11 biomarkers to be significantly altered. Among these, 8 biomarkers were significantly increased in patients developing mutations on D2, and included angiogenic growth factors (bFGF, PlGF, and VEGF-D), angiogenic factors' receptors (Tie-2 and Flt-1), and drivers of healing responses through macrophages (MCP-2 and MCP-3) (**Figure 4A**). The 4 biomarkers that were significantly downregulated were acute-phase inflammatory marker SAA, neutrophil chemokine IL-8, immunomodulatory marker IL-10, as well as M-CSF, a key cytokine involved in macrophage differentiation that enhances the inflammatory response of primed macrophages. Interestingly, after 48 hours of mAb infusion, the only cytokines observed to be significantly altered (n = 8) were those that were also significantly altered on D0 (**Figure 4B**). By D7, several of these mutation-associated cytokines stayed altered (**Figure 4C**). These data suggest that, firstly, therapeutic mAbs do not substantially alter cytokine profiles in mildly ill COVID-19 patients, and secondly, cytokines identified to be linked to de novo S RBD mutation development are quite robust. AUROC data were further validated with random forest classification, which identified a signature consisting of SAA, Tie-2, bFGF, and M-CSF that correctly identified patients with de novo S RBD mutations with high predictability (mean ROC of 96%). While C-reactive protein (CRP) on its own missed statistical significance with AUROC analysis, replacing CRP with SAA did not change the accuracy of the model, likely because of high degree of colinearity identified between CRP and SAA (Pearson's $r = 0.937$, $P < 0.001$; **Figure 4D**). This signature was further independently tested on 19 patients, 8 of whom received sotrovimab and 11 of whom received tixagevimab/cilgavimab. Patient characteristics are described in **Figure 5A**. One patient each receiving sotrovimab or tixagevimab/cilgavimab developed S RBD mutations within 7 days of receiving mAb therapy. All 19 samples were correctly classified utilizing the CIB-based signature, both by random forest classification (AUROC = 1) or a binomial logistic regression model ($\chi 2 = 12.787$, $n = 19$, $df = 4$, $P < 0.012$; **Figure 5, B and C**). Remarkably, bFGF levels alone led to a 100% correct classification, with mutation carriers having bFGF levels of 23.7 pg/mL or higher (n = 2, range 23.7–34.4 pg/mL) and non–mutation carriers with levels of 19 pg/mL or lower (n = 17, average 5.5 pg/mL, range 0.5–19 pg/mL). These data not only suggest that a diminished proinflammatory and homeostatic cytokine immune milieu could facilitate development of de novo S RBD mutations, but also describe a CIB profile present before mAb administration that predicts development of escape mutations against therapeutic mAbs for SARS-CoV-19 in high-risk patients with high accuracy.

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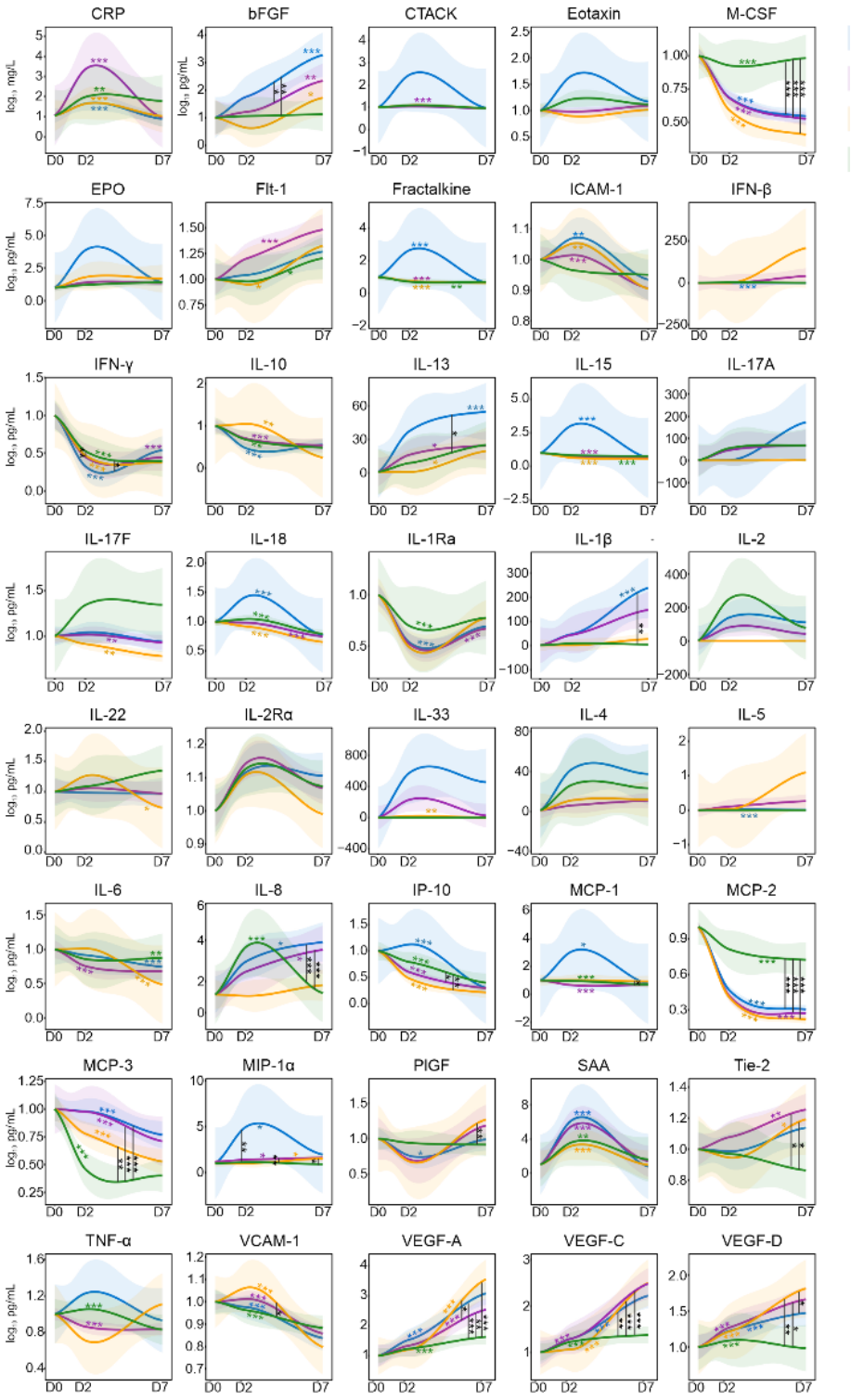

Figure 3: Temporal evolution of circulating immune-related biomarkers (CIBs) in patients receiving bamlanivimab, bamlanivimab/etesevimab, casirivimab/imdevimab, or sotrovimab therapy. Time is represented as days after mAb therapy (D0, D2, and D7). Cross-sectional and longitudinal statistical comparisons were performed using Mann-Whitney followed by Bonferroni post-hoc correction. Lines represent smoothed conditional means for studied timepoints and shaded area display 95% confidence intervals for all measured timepoints. Colored asterisks in the graph refer to the significance of the slope from the 4 separate regression lines. Vertical lines with asterisks represent the significance of the pairwise comparison between the slopes in bamlanivimab, bamlanivimab/etesevimab, casirivimab/imdevimab, and sotrovimab therapy groups. D0: sample collected prior to mAb infusion. D2: 2 ± 1 days after mAb infusion. D7: 7 ± 2 days after mAb infusion. D28: 28 ± 4 days after mAb infusion. *: $p < 0.05$. **: $p < 0.01$. ***: $p < 0.001$.

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Up (D0)	Fold change	p-value
Tie-2	2.0	<0.001
Flt-1	1.7	0.005
bFGF	3.9	0.001
VEGF-D	1.5	0.032
PlGF	1.6	0.045
MCP-2	1.4	0.013
MCP-3	2.2	0.014

Down (D0)	Fold change	p-value
SAA	0.6	0.038
IL-8	0.5	0.036
M-CSF	0.5	0.012
IL-10	0.2	0.023

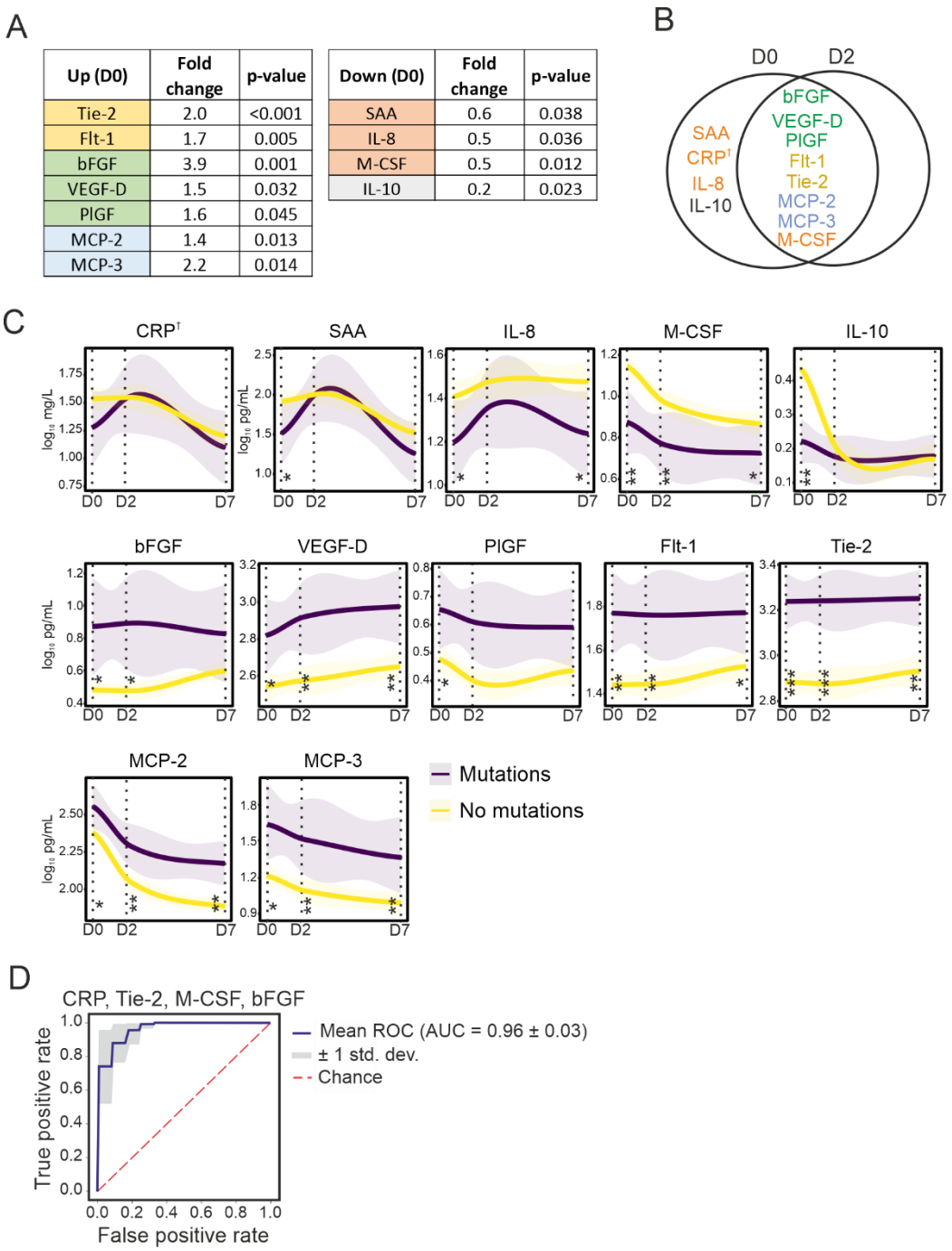

Figure 4: Circulating immune-related biomarkers (CIB) in COVD-19 patients receiving mAb therapy. (A) Several CIBs are significantly up- or downregulated at D0 in COVID-19 patients that developed SARS-CoV-2 Spike RBD mutations after administration of mAb treatments, compared to who did not. **(B)** Eleven CIBs were significantly altered at D0 in patients with de novo Spike RBD mutations, for which the majority (n = 8) were also altered at D2. **(C)** Temporal evolution of CIBs altered in patients with or without de novo mutations, receiving mAb therapy through day 7 after treatment. Lines represent smoothed conditional means and shaded areas display 95% confidence intervals for all measured timepoints. P-values refer to significance of the slope of the regression lines. Vertical lines with asterisks represent the significant difference between CIB levels at the specified timepoints. **(D)** Receiving operator characteristic (ROC) curve in a random forest classifier model with Synthetic Minority Oversampling Technique (SMOTE) for the prediction of mutation versus no-mutation are depicted for D0. *: $p < 0.05$. **: $p < 0.01$. ***: $p < 0.001$. †: not significant.

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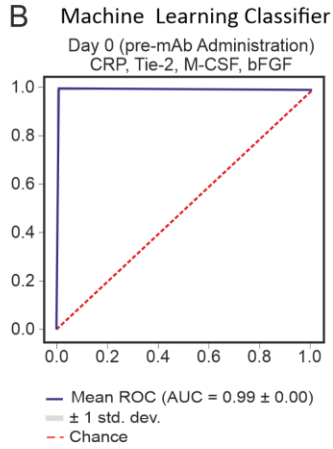
Patient characteristics	Non mutation (n=17)	Mutation carriers (n=2)	P-value
Sotrovimab therapy (Spike RBD amino acid substitution)	**7 (41.2)**	**1 (K346R, L371S, P373S, F375S)**	**NA**
Tixagevimab/cilgavimab therapy (Spike RBD amino acid substitution)	**10 (58.8)**	**1 (R408S)**	**NA**
Male (%)	9 (52.9)	1 (50.0)	NS
Age (mean, IQR or range)*	66 (62-75)	62 (51-72)	NS
< 65 years	57 (52-64)	51 (51-51)	NS
≥ 65 years	76 (72-79)	72 (72-72)	NS
BMI (median, IQR or range)	28 (25-31)	22 (21-24)	NS
WHO progression severity scale – At enrolment (mean, IQR or range)*	2 (2-3)	3 (2-3)	NS
WHO progression severity scale – Worst (mean, IQR or range)*	2 (2-3)	3 (2-3)	NS
Days from symptoms onset to mAb infusion (mean, IQR or range)*	2 (1-3)	3 (2-3)	NS
sO2 % (mean, IQR or range)*	97 (96-98)	96 (94-97)	NS
Anti-SARS-CoV-2 vaccination (>2 weeks post-dose, ≥2 doses, %)	16 (94.1)	2 (100)	NS
Ongoing COVID-related therapy (prednisone, azithromycin, amoxicillin/clavulanate)	0 (0.0)	0 (0.0)	NS
Immunocompromising condition (%)	7 (41.2)	2 (100)	NS
Solid organ cancer (with ongoing therapy/ongoing stopped < 6 mo) (%)	1 (5.9)	0 (0.0)	NS
Hematologic cancer (with ongoing CHT/ongoing stopped < 6 mo) (%)	3 (17.6)	1 (50.0)	NS
Solid organ transplant recipients (%)	0 (0.0)	0 (0.0)	NS
Immunological diseases requiring immunosuppressive agents (%)	4 (23.5)	1 (50.0)	NS
Other comorbidities			
Diabetes (with or without damage) (%)	2 (11.8)	0 (0.0)	NS
Cardiovascular disease (ischemic/arrythmia/hypertension) (%)	9 (52.9)	0 (0.0)	NS
Chronic renal failure (with or without need of dialysis) (%)	0 (0.0)	0 (0.0)	NS
Chronic pulmonary diseases (%)	5 (29.4)	1 (50.0)	NS
Any neurological/vascular disease (%)	1 (5.9)	0 (0.0)	NS
Viral variant			
BA.1/Omicron (%)	3 (17.6)	0 (0.0)	NS
BA.1+R346K/Omicron (%)	1 (5.9)	1 (50.0)	
BA.2/Omicron (%)	7 (41.2)	0 (0.0)	
BA.4/Omicron (%)	1 (5.9)	0 (0.0)	
BA.5/Omicron (%)	2 (11.8)	0 (0.0)	
BE.1/Omicron (%)	1 (5.9)	1 (50.0)	

*: where n=2, ranges are displayed;NA: not applicable; NS: non-significant,

B



C

Regression classification

		Mutations		Percentage Correct
		No	Yes	
Mutations	No	17	0	100
	Yes	0	2	100
Overall Percentage				100

Figure 5: Out-of-sample performance of circulating immune-related biomarkers (CIBs) predicting *de novo* SARS-CoV-2 Spike RBD mutations in COVID-19 patients receiving mAb therapy. (A) Clinical characteristics of the enrolled patients for CIB validation. Statistical assessments of categorical and continuous variables were assessed across mAb therapy groups using chi-square tests of independence and analysis of variance (ANOVA), respectively. IQR: interquartile range. mo: months. (B) Utilizing random forest classification with SMOTE analysis based on a CIB panel comprising 4 biomarkers (CRP, Tie-2, M-CSF, and bFGF) before mAb treatment predicted de novo Spike RBD mutation development with AUROC of 0.99 within seven days of treatment. (C) Binomial logistic regression also predicted patients with or without de novo Spike RBD mutations with 100% accuracy.

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Output

These results have been presented as an oral presentation at the ECCMID conference 2023

Akshita Gupta, Angelina Konnova, Mathias Smet, Matilda Berkell, Alessia Savoldi, Matteo Morra, Vincent Van Averbeke, Fien De Winter, Denise Peserico, Elisa Danese, An Hotterbeekx, Elda Righi, Pasquale De Nardo, Evelina Tacconelli, Surbhi Malhotra-Kumar, Samir Kumar-Singh, *Host immunological responses facilitate development of SARS-CoV-2 mutations in patients receiving monoclonal antibody treatments*, Abstract 04724

And have been published in Journal of Clinical Investigation

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