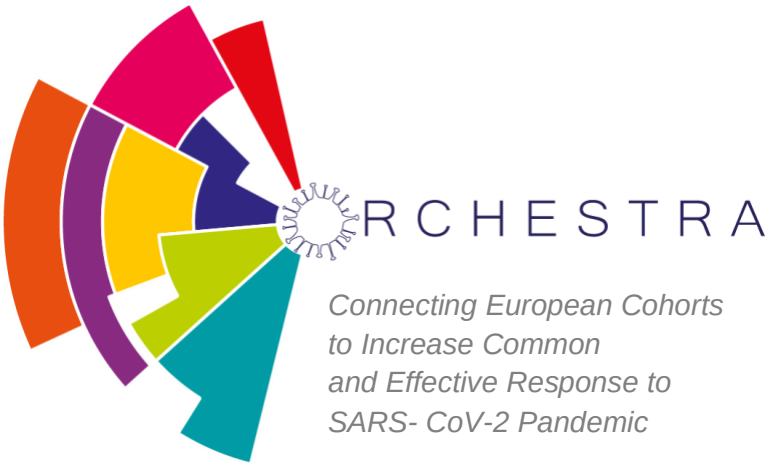

DELIVERABLE

WPN°6_Deliverable N°6.5,

Report on cytokine chemokine analysis in COVID-19 patients from retrospective cohorts

Prof Dr Samir Kumar-Singh

WP6_ D6.5

University of Antwerp (UA)

Project Classification

Project Acronym:	*ORCHESTRA*
Project Title:	*Connecting European Cohorts to Increase Common and Effective Response to SARS- CoV-2 Pandemic*
Coordinator:	*UNIVR*
Grant Agreement Number:	*101016167*
Funding Scheme:	*Horizon 2020*
Start:	*1st December 2020*
Duration:	*36 months*
Website:	*www.orchestra-cohort.eu*
Email:	*info@orchestra.eu*

Document Classification

WP No:	*WP6*
Deliverable No:	*D6.5*
Title:	*Report on cytokine chemokine analysis in COVID-19 patients from retrospective cohorts*
Lead Beneficiary:	*UAntwerp*
Other Involved Beneficiaries:	
Nature:	*Report*
Dissemination Level:	*Public*
Due Delivery Date:	*31.08.2021 (M9)*
Submission Date:	*31.10.2021 (M11)*
Justification of delay:	*Samples with database arrived 6/10/2021*
Status:	*Final*
Version:	*1.0*
Author(s):	*Samir Kumar-Singh, An Hotterbeekx, Surbhi Malhotra, Matilda Berkell*

History of Changes

Version	***Date***	***Created/Modified by***
0.1	*29/10/2021*	*An Hotterbeekx, Samir Kumar-Singh (UANT)*
0.2	*17/11/2021*	*An Hotterbeekx, Samir Kumar-Singh (UANT)*
0.3	*27/12/2021*	*Benoit Visseaux (INSERM)*
1.0	*27/01/2022*	*An Hotterbeekx (UANT)*

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

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 390 samples from 192 patients included from retrospective cohorts covering the year 2020. The aim is to identify biomarkers based on CCG for the prediction of acute respiratory distress syndrome (ARDS) in hospitalized patients based both on the earliest hospitalization timepoint close to the study enrollment timepoint as well as from any subsequent timepoints but before the development of ARDS. In addition, the aim is to compare CCG alterations occurring during the peak of ARDS as well as any long-term effect in patients (up to 3 months after hospital discharge) in COVID-19 patients with or without ARDS.

Dissemination level: Public

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Core content

All samples of interest collected before 30/10/2021 were analyzed. These samples originated from Inserm (Hôpital Bichat Claude Bernard). Twenty-four cytokines, chemokines and growth factors were measured using the Meso Scale Discovery (MSD, MD, USA) platform.

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

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 24 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)-β.

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).

Description of the cohort and selected samples

Selection of patients and samples

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

Cohort name	Country	Type of patients	Samples included	CCG's measured	Detection method
FrenchCOVID	France	Hospitalized	390	24 for each sample	Meso-scale discovery assay

To identify early biomarkers predicting ARDS and disease outcome and to investigate changes after ARDS development, the following patients and samples were selected:

- The **no ARDS group** consists of hospitalized patients with COVID-19 who did not develop ARDS and is used compare CCG alterations in the ARDS group. Within this group the following samples were studied:

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

- The baseline sample (D1). In D1 sample was not available, day 3 after hospital admission (D3) was studied as baseline sample.
- The timepoint closest to the middle of the hospital stay (D-mid).
- The sample 3 months after discharge (M3)

- For patients who **developed ARDS,** the following samples were selected:
  - The baseline sample (D1)
  - The sample at ARDS diagnosis if available (ARDS)
  - The sample closest before ARDS development (Pre-ARDS)
  - The sample at the peak ARDS severity (2-3 days after the diagnosis) (post ARDS)
  - In case a patient was hospitalized with ARDS, the D1 sample was selected (D1 ARDS) along with a post ARDS sample close to the diagnosis (post-ARDS)
  - The sample 3 months after discharge (M3)

Table 2. *Selection patients and samples tested for cytokines among individuals without ARDS. In total 24 cytokines were measured in each sample unless indicated otherwise. D1: admission sample; Baseline D3: D3 was the earliest sample available and considered as baseline; D-mid: timepoint closest to the mid-hospital stay; DD: discharge day; M3: Sample after 3 months.*

Group	D1	Baseline D3	Dmid	DD	M3
NO_ARDS	71	22	46	2	56
s_001-0234		1			1
s_001-0246		1			1
s_001-0261		1			1
s_001-0263		1			1
s_001-0264		1			1[a]
s_001-0272		1			1
s_001-0274		1			1
s_001-0277		1			1
s_001-0281		1			1
s_001-0289	1				1
s_001-0291		1			1
s_002-0177			1		
s_004-0001	1		1		1
s_004-0002		1			1
s_004-0007	1			1	1
s_006-0006	1		1		1
s_006-0008	1		1		1
s_009-0005		1			1
s_015-0005		1			1

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

s_015-0021	1		1		1
s_019-0005		1[b]			1[c]
s_019-0009	1				1
s_019-0039		1			1
s_020-0065	1				
s_020-0067				1	
s_022-0001	1				
s_022-0002	1		1		
s_022-0003	1				
s_022-0008		1			1
s_022-0009	1				
s_022-0011	1		1		1
s_023-0002		1			1
s_023-0003		1			1
s_023-0010	1		1		1
s_023-0014		1			1
s_023-0016	1		1		1
s_023-0018	1		1		1
s_023-0024	1				
s_023-0025	1				1
s_023-0027		1			1
s_023-0028	1				1
s_023-0029	1		1		1
s_023-0032	1				1[a]
s_023-0034	1				1
s_023-0039			1		1
s_023-0060	1		1		1
s_023-0076			1		1
s_023-0085	1		1		1
s_023-0087	1		1		
s_023-0088	1		1		1
s_023-0090	1		1		1
s_023-0094	1		1		1
s_023-0100	1		1		
s_023-0106	1				
s_023-0107	1				
s_023-0111	1		1		
s_023-0114	1				
s_023-0115	1		1		
s_023-0116	1				
s_023-0121	1				
s_039-0003	1		1		
s_039-0015	1		1		
s_039-0019	1		1		
s_039-0042	1		1		
s_039-0057	1		1		

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

s_039-0065	*1*		*1*		
s_039-0071	*1*		*1*		
s_047-0001	*1*		*1*		*1*
s_047-0012	*1*		*1*		
s_047-0048	*1*		*1*		*1*
s_053-0005	*1*				*1*
s_053-0014	*1*				
s_055-0037	*1*		*1*		*1*
s_055-0039					*1*[d]
s_055-0137					*1*
s_060-0034	*1*				
s_060-0036	*1*				
s_062-0006	*1*				
s_062-0008	*1*		*1*		
s_062-0010	*1*				
s_062-0012	*1*				
s_062-0013	*1*		*1*		
s_062-0014	*1*				
s_076-0001		*1*			*1*
s_076-0004	*1*		*1*		*1*
s_082-0001	*1*		*1*		*1*
s_086-0002	*1*				*1*
s_086-0005					*1*[b]
s_089-0003	*1*		*1*		
s_089-0009	*1*		*1*		
s_089-0010	*1*		*1*		
s_091-0005	*1*		*1*		
s_091-0006	*1*		*1*		
s_091-0007	*1*		*1*		
s_091-0009	*1*		*1*		
s_091-0011	*1*		*1*		
s_091-0016	*1*				
s_091-0023		*1*			*1*
s_092-1003					*1*
s_112-0003	*1*		*1*		
s_112-0008	*1*		*1*		
DEATH NO_ARDS	***2***	***1***			
s_002-0003		*1*			
s_011-0004	*1*				
s_022-0007	*1*				

[a]Only 14 cytkines measured due to insufficient sample volume
[b]Only 5 cytkines measured due to insufficient sample volume
[c]Only 19 cytkines measured due to insufficient sample volume
[d]Only 20 cytkines measured due to insufficient sample volume

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Table 3. *Selection patients and samples tested for cytokines among patients who developed ARDS and survived after hospital discharge. In total 24 cytokines were measured in each sample unless indicated otherwise. D1 preARDS: first sample at admission without ARDS; D1 ARDS: admission with ARDS; PreARDS: closest sample before ARDS; PostARDS: closest sample after ARDS; ARDS 3M: sample after 3 months*

Group	D1 ARDS	D1 preARDS	PreARDS	ARDS	PostARDS	ARDS M3
ARDS	**17**	**19**	**14**	**6**	**47**	**21**
s_001-0292			1			
s_002-0037	1				2	1
s_002-0067			1		1	
s_002-0079			2			
s_002-0152		1				1
s_004-0003					1	1
s_016-0003					1	1[a]
s_016-0009					1	
s_022-0020	1				2	
s_023-0001		1			1	1
s_023-0005				1		
s_023-0006				1	1	
s_023-0008			1		1	1
s_023-0013						1
s_023-0021		1				
s_023-0030					1	1
s_023-0031		1			1	1
s_023-0033	1				1	1
s_023-0037	1					1
s_023-0040		1				1
s_023-0075	1				1	1
s_023-0078		1	2			1
s_023-0091	1				1	1
s_023-0098	1				1	1
s_023-0099		1			1[a]	1
s_023-0102		1			1	
s_023-0104	1[b]					
s_023-0113		1			1	
s_023-0118		1			1	
s_031-0022				1	1	
s_039-0012	1					
s_039-0013	1				1	
s_039-0023	1				1	
s_039-0024	1				1	
s_039-0030			1		1	
s_039-0034					1	
s_039-0050				1	1	

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

s_039-0054		*1*	*1*			
s_039-0055		*1*			*1*	
s_039-0060		*1*			*2*	
s_039-0070	*1*				*1[c]*	
s_039-0072				*1*	*1*	
s_039-0075		*1*	*1*			
s_039-0076	*1*				*2*	
s_039-0077					*1*	
s_039-0079					*1*	
s_039-0080					*1*	
s_039-1006			*1*		*1*	
s_047-0013		*1*	*2*			*1*
s_054-0011					*1*	*1*
s_055-0138		*1*			*1*	*1*
s_060-0031			*1*			
s_062-0009		*1*			*1*	
s_063-0022		*1*			*1[a]*	
s_063-0144	*1*				*1*	
s_063-0150	*1*				*1*	
s_076-0003	*1*					*1*
s_091-0018		*1*		*1*	*2*	

[a]Only 19 cytokines measured due to insufficient sample volume
[b]Only 24 cytokines measured due to technical problem
[c]Only 5 cytokines measured due to insufficient sample volume

Table 4. *Selection patients and samples tested for cytokines among patients who developed ARDS and died during hospitalization. In total 24 cytokines were measured in each sample. D1 pre ARDS: first sample at admission without ARDS; D1 ARDS: admission with ARDS; PreARDS: closest sample before ARDS; PostARDS: closest sample after ARDS; ARDS 3M: sample after 3 months.*

Group	***D1 ARDS***	***D1 preARDS***	***PreARDS***	***ARDS***	***PostARDS***	***ARDS M3***
DEATH ARDS	***10***	***12***	***8***	***4***	***28***	***0***
s_001-0175		*1*			*1*	
s_002-0007			*1*	*1*	*1*	
s_002-0026				*1*	*1*	
s_002-0031			*1*	*1*	*2*	
s_006-0002					*2*	
s_015-0002			*1*		*1*	
s_020-0009		*1*				
s_022-0010		*1*				
s_023-0012		*1*				
s_031-0005					*1*	
s_031-0021					*1*	

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

s_031-0029	*1*				*1*	
s_041-0016		*1*	*2*		*1*	
s_041-0019		*1*	*2*		*1*	
s_041-3001		*1*			*1*	
s_041-3002		*1*			*1*	
s_041-3007		*1*			*1*	
s_041-4006	*1*				*1*	
s_041-4011	*1*				*1*	
s_041-4015	*1*				*1*	
s_041-4018	*1*				*1*	
s_041-4020	*1*				*1*	
s_041-4024	*1*				*1*	
s_055-0134	*1*				*1*	
s_063-0065		*1*		*1*		
s_063-0066		*1*			*1*	
s_063-0092		*1*	*1*		*2*	
s_063-0096	*1*				*1*	
s_063-0132	*1*				*1*	

Statistical analysis

All data were analysed using SPSS v27, R (version R4.0.4) and Metaboanalyst 5.0 (https://www.metaboanalyst.ca/). Data that were not normally distributed, were naturally log transformed before independent sample t-test was performed to compare CCG production in different groups. Data were uploaded in Metaboanalyst 5.0 for the biomarker discovery. Prior to analysis, missing values were estimated using KNN (sample-wise). Data are Log transformed and auto-scaling was implemented before sample normalization. Receiver Operating Characteristic (ROC) curve analysis was performed to identify potential biomarkers. Only CCGs with an Area Under the Curve (AUC) >70% were considered.

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Preliminary results

Identification of CCGs differentially produced during ARDS

We first investigated which CCGs were differentially expressed during ARDS compared to patient hospitalized with COVID-19 but without ARDS. To do so, we compared 111 samples of 72 patients who had ARDS with 140 samples from 96 patients who did not develop ARDS. Samples from ARDS patients include the D1 sample from patients admitted to the hospital with ARDS (N=27), samples on the day ARDS was diagnosed (N=9) and samples just after the ARDS diagnosis (N=75). Samples from non-ARDS patients include samples close to hospital admission (N=94) and samples taken at mid hospital stay (N=46).

The results are shown in **Figure 1** (see next page)

Briefly, the significantly upregulated CCGs during ARDS are the pro-inflammatory cytokines IL-6 ($p<0.001$) and IL-8 ($p<0.001$), interferon-related cytokines IP-10 ($p<0.001$) and IL-18 ($p<0.001$), Th2-related cytokines IL-4 ($p<0.05$) and IL-9 ($p<0.05$), Treg-related cytokine IL-10 ($p<0.001$), colony stimulating factors M-CSF ($p<0.001$) and GM-CSF ($p<0.001$), chemoattractants TSLP ($p<0.001$), MCP-3 ($p<0.001$) and fractalkine ($p<0.001$). Furthermore, Th17-related cytokine IL-17F ($p<0.05$) is significantly downregulated during ARDS.

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

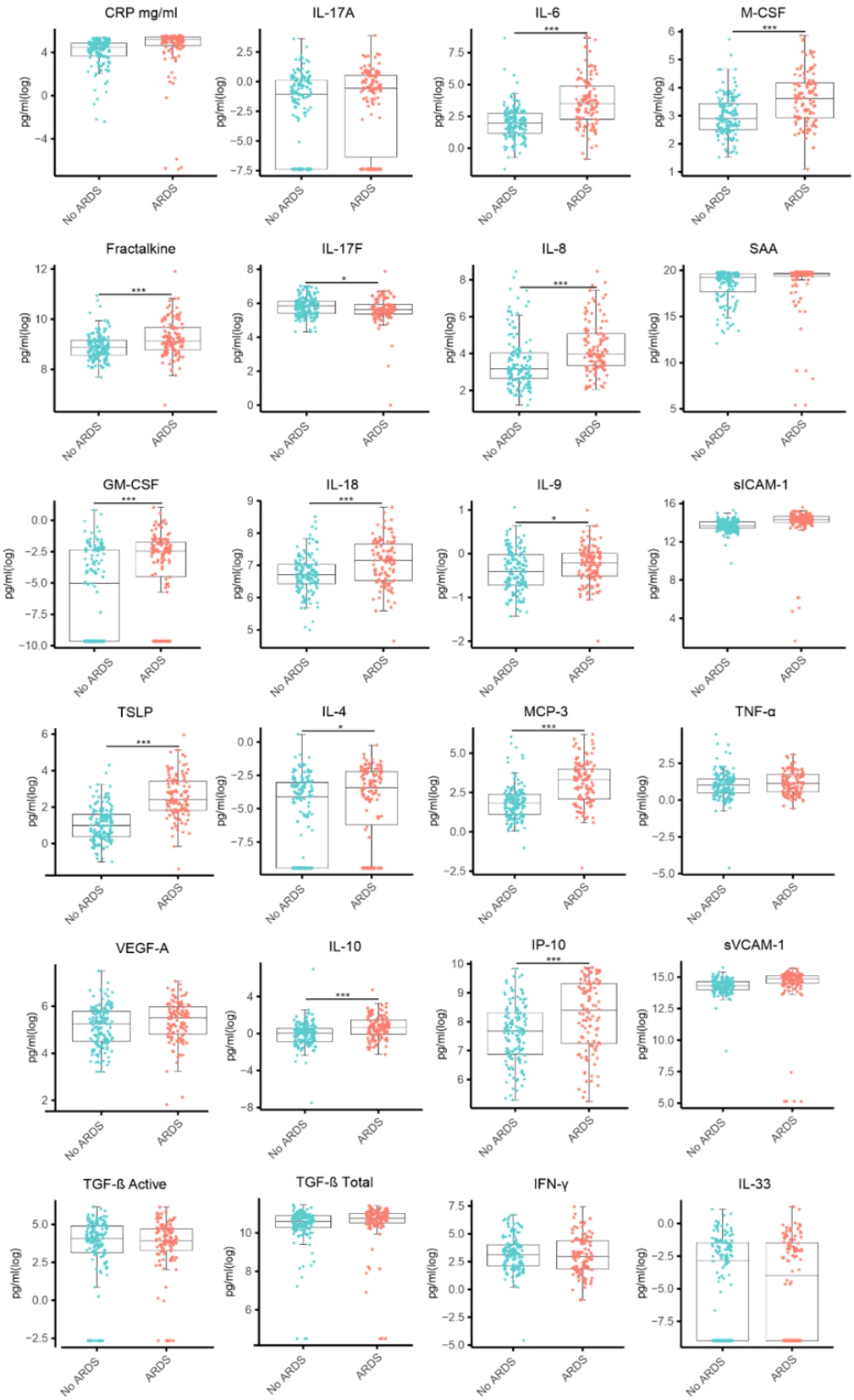

Figure 1: *CCGs in serum of patients without and with ARDS at the time of ARDS. ***p<0.001; **p<0.01; *p<0.05.*

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Identification of potential biomarkers to predict ARDS development

Next, we aimed to identify CCG biomarkers to predict ARDS development in patients who are admitted in the hospital with COVID-19 at the earliest possible timepoint. To do so, we compared the cytokine profiles of ARDS (N=31) and non-ARDS (N=94) patients in the baseline sample. These samples were taken at the day of hospitalization (D1) or, if no D1 sample was available, at day 3 of hospitalization (this was the case for 22/94 non-ARDS patients).

At this early timepoint, the pro-inflammatory cytokine IL-6 ($p<0.001$), interferon-related cytokines IFN-γ ($P<0.01$) and IP-10 ($p<0.001$), Th17-related cytokine IL-17F ($p<0.05$), Th2-related cytokines IL-33 ($p<0.05$) and IL-9 ($p<0.01$), Treg-related cytokine IL-10 ($p<0.01$), colony stimulating factors M-CSF ($p<0.01$) and GM-CSF ($p<0.01$), chemoattractants MCP-3 ($p<0.001$), TSLP ($p<0.001$) and fractalkine ($p<0.01$) and adhesion molecules sICAM-1 ($p<0.05$) and sVCAM-1 ($p<0.05$) were significantly upregulated in persons who developed ARDS later during the course of hospitalization (**Figure 2**).

When analyzing data for early predictive markers for ARDS, ROC curve analysis showed 5 cytokines with a predictive value >70% (**Table 5**). Of these, IP-10, IL-6, MCP-3 and IL-10 have been described before as predictive markers for ARDS whereas the role of TSLP has not yet been explored.

Table 5: *ROC curve analysis of CCG biomarkers predicting the development of ARDS at the time of hospitalization. Only cytokines/chemokines with and area under the curve (AUC) >0.70 were considered.*

Cytokine	***AUC***	***T-test***	***Log2 Fold change***
IP-10	*0.83*	*0.002*	*1.391*
IL-6	*0.77*	*<0.000*	*0.007*
MCP-3	*0.77*	*0.001*	*0.754*
TSLP	*0.75*	*0.001*	*1.048*
IL-10	*0.73*	*0.006*	*-1.453*

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

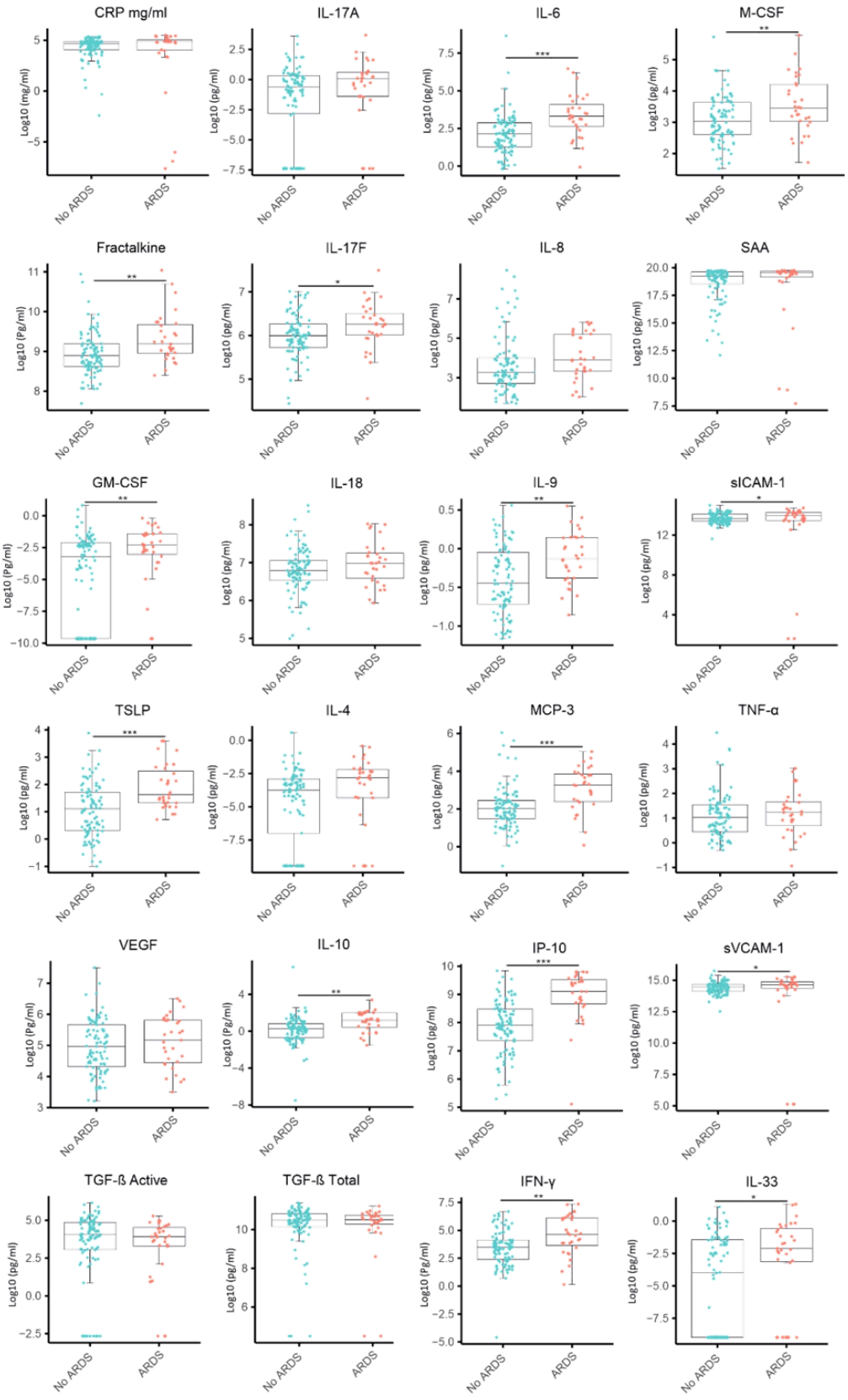

Figure 2: *CCGs in serum of patients without and with ARDS at the time of inclusion. ***p<0.001; **p<0.01; *p<0.05.*

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

We further aimed to identify CCGs biomarkers to predict ARDS development in patients hospitalized with COVID-19 at any timepoint during hospitalization before ARDS diagnosis. To do so, we compared all available samples before ARDS diagnosis (N=53 samples from 35 patients) with samples taken from 131 patients who did not develop ARDS at the time of hospitalization (N=94) and/or during the middle of the hospitalization (N=46).

Here the pro-inflammatory cytokine IL-6 ($p<0.001$), interferon-related cytokines IFN-γ ($P<0.01$) and IP-10 ($p<0.001$), Th2-related cytokines, IL-4 ($p<0.01$), IL-9 ($p<0.05$), Treg-related cytokine IL-10 ($p<0.001$), colony stimulating factors M-CSF ($p<0.001$) and GM-CSF ($p<0.001$), chemoattractants TSLP ($p<0.001$), MCP-3 ($p<0.001$) and fractalkine ($p<0.001$) were significantly upregulated in persons who developed ARDS later during the course of hospitalization (**Figure 3**).

When looking for predictive markers for ARDS, ROC curve analysis showed 8 cytokines with a predictive value >70% (**Table 6**). Of these, the four cytokines/chemokines with the highest predictive value are the same as described above when only considering the earliest timepoint, but the top three is in a different order whereas the predictive value of TSLP remains the same (0.75).

Table 6: *ROC curve analysis of CCG biomarkers predicting the development of ARDS at any timepoint during hospitalization. Only CCGs with and area under the curve (AUC) >0.70 were considered.*

Cytokine	***AUC***	***T-test***	***Log2 Fold change***
MCP-3	*0.81*	*0.00*	*1.01*
IP-10	*0.78*	*0.00*	*1.34*
IL-6	*0.76*	*0.00*	*-0.08*
TSLP	*0.75*	*0.00*	*0.98*
GM-CSF	*0.71*	*0.00*	*0.67*
IL-10	*0.70*	*0.00*	*-1.31*
sVCAM-1	*0.70*	*0.62*	*0.46*
CRP	*0.70*	*0.56*	*0.59*

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

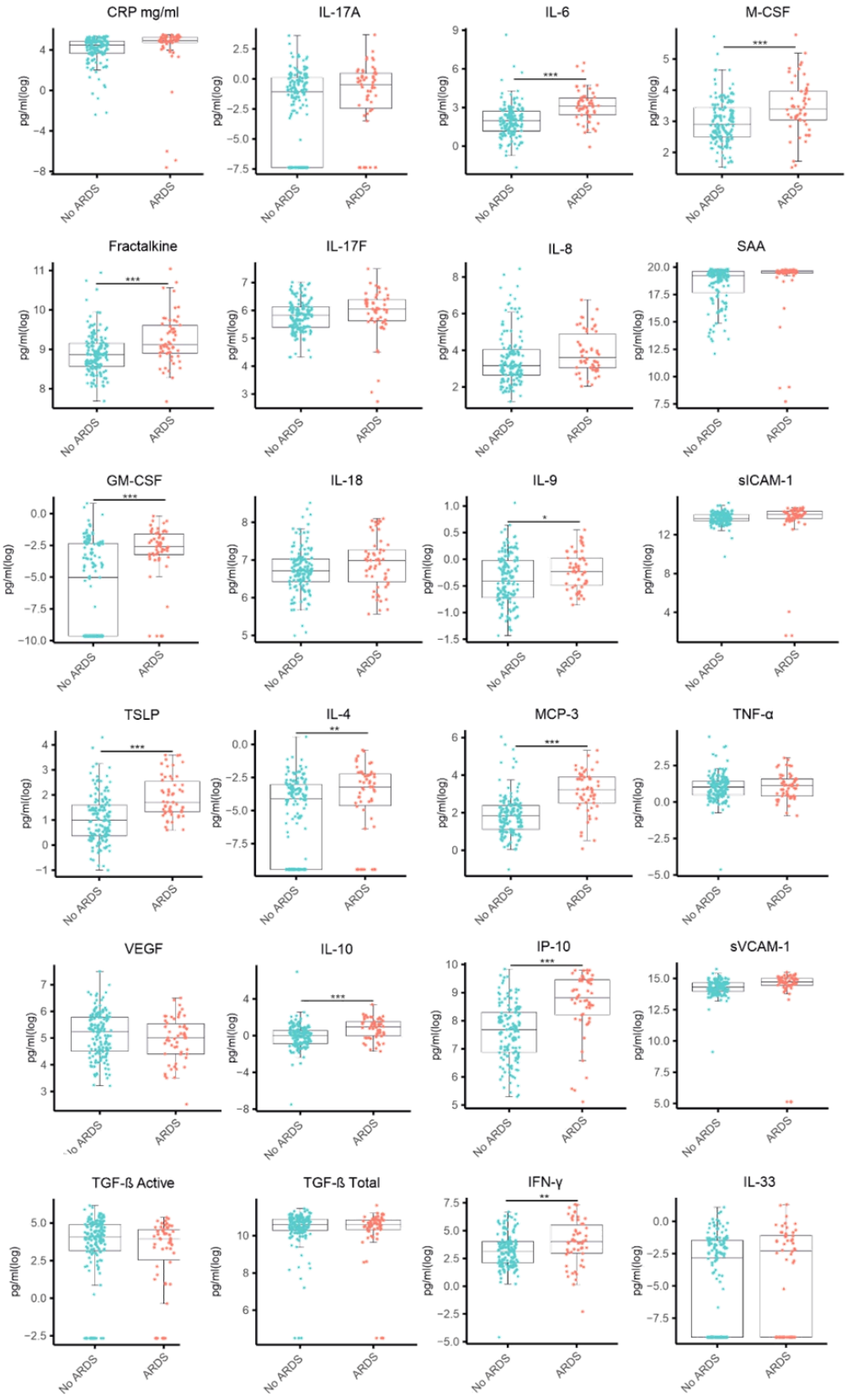

Figure 3: *CCGs in serum of patients without and with ARDS at any time before the development of ARDS. ***p<0.001; **p<0.01; *p<0.05.*

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167

Acknowledgments

The WP6 want to acknowledge the cohorts that provided the samples and associated clinical data:

FrenchCOVID cohort: Cédric Laouénan, Sarah Tubiana, Patrick Rossignol, France Mentré, Lila Bouadma, Isabelle Hoffmann, Xavier Duval, Catherine Chirouze, Jade Ghosn

ORCHESTRA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101016167