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Role of immune mediators in predicting hospitalization of SARS-CoV-2 positive patients

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ABSTRACT

Background: Several immune mediators (IM) including cytokines, chemokines, and their receptors have been suggested to play a role in COVID-19 pathophysiology and severity.
Aim: To determine if early IM profiles are predictive of clinical outcome and which of the IMs tested possess the most clinical utility.
Methods: A custom bead-based multiplex assay was used to measure IM concentrations in a cohort of SARS-CoV-2 PCR positive patients (n = 326) with varying disease severities as determined by hospitalization status, length of hospital stay, and survival. Patient groups were compared, and clinical utility was assessed. Correlation plots were constructed to determine if significant relationships exist between the IMs in the setting of COVID-19.
Results: In PCR positive SARS-CoV-2 patients, IL-6 was the best predictor of the need for hospitalization and length of stay. Additionally, MCP-1 and sIL-2Rα were moderate predictors of the need for hospitalization. Hospitalized PCR positive SARS-CoV-2 patients displayed a notable correlation between sIL-2Rα and IL-18 (Spearman's $\rho = 0.48$, P=<0.0001).
Conclusions: IM profiles between non-hospitalized and hospitalized patients were distinct. IL-6 was the best predictor of COVID-19 severity among all the IMs tested.

1. Introduction

COVID-19 has placed significant strain on hospitals worldwide. Limited resources and hospital inpatient capacity for patients with severe SARS-CoV-2 infections have become a major obstacle during the pandemic. Given the highly variable presentation between patients, accurately predicting the course of the disease is vital to ensure proper planning and allocation of resources. The role of cytokines has been an important topic, as severe COVID-19 cases are often accompanied by overactive immune responses [1]. The high degree of variability regarding initial viral dose, viral genotype, general health, and genetic predispositions between patients have made it challenging to determine clinically useful cytokine patterns or gain mechanistic insight into disease pathogenesis.

SARS-CoV-2 infections have a wide range of presentations, with the most severe cases involving the infection of the alveolar pneumocytes in the lower respiratory tract [2]. While some individuals can clear the infection, other, often older individuals and/or those with

Abbreviations: IM, Immune Mediator; LLOQ, Lower Limit of Quantification; AMR, Analytic Measuring Range; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; COVID-19, Coronavirus disease 2019; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IFNα, IFNβ, IFNγ, Interferon α, β, γ; IL, Interleukin; IL-1β, Interleukin 1 beta; sIL-2Rα, Interleukin 2 receptor α (soluble); IL-6, Interleukin 6; sIL-6Rα, Interleukin 6 receptor α (soluble); IL-10, Interleukin 10; IL-18, Interleukin 18; MCP-1, Monocyte chemoattractant protein-1; MIP-1α, Macrophage inflammatory protein 1α; TNF, TNF-α, Tumor necrosis factor-alpha; VEGF, Vascular endothelial growth factor; IP-10, CXCL-10, Interferon gamma-induced protein 10; vWF-A2, von Willebrand factor A2 domain; FGF, Fibroblast growth factor; G-CSF, Granulocyte colony-stimulating factor; PDGF, Platelet-derived growth factor; EN-RAGE/S100A12, Extracellular newly identified receptor for advanced glycation end products binding protein; LIF, Leukemia inhibitory factor; CLIR, Collaborative Laboratory Integrated Reports.

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comorbidities, experience severe pneumonia which can lead to systemic inflammation, organ failure, or death [3]. In these severe cases, there appears to be major dysregulation of the immune response with abnormal concentrations of cytokines, chemokines, and their receptors. Numerous studies have described cytokine elevations in COVID-19, with elevations in IL-1β, IL-1Rα, IL-7, IL-8, IL-9, IL-10, basic FGF, G-CSF, GM-CSF, IFNγ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, TNF, and VEGF concentrations in both intensive care unit (ICU) patients and non-ICU patients compared to healthy adults [3]. In another study, the expression of interleukin IL-6, IL-10, and IP-10 was suggested to be closely correlated with disease progression [4]. To our knowledge, very few groups have been able to examine non-hospitalized symptomatic SARS-CoV-2 positive individuals with regards to immune mediator (IM) levels in plasma [5,6].

In the present study, we measured 14 analytes (MCP-1, MIP-1α, soluble IL-2 receptor α (sIL-2Rα), GM-CSF, IFNα, IFNβ, IFNγ, IL-1β, IL-6, soluble IL-6 receptor α (sIL-6Rα), VEGF, IL-10, IL-18, and TNF) potentially related to cytokine release syndrome. These analytes were chosen to sample both pro and anti-inflammatory cytokines, as well as soluble receptors previously reported to be affected (elevated or suppressed) in patients with cytokine release syndrome due to CAR T-cell therapies, SARS-CoV-2, MERS, SARS, and ARDS [3,7–10]. GM-CSF and sIL-6Rα were included as these were targets of early cytokine modulator therapies for COVID-19 [11–13]. The objective of this study was to determine if early IM measurements in PCR positive SARS-CoV-2 patients would be predictive of disease outcome (need for hospitalization or not). Within the hospitalized cohort, we aimed to determine whether individual IMs or a combination thereof could distinguish those patients with a shorter hospital stay (<10 days) from those with a longer stay (≥10 days) or disease mortality. Additionally, relationships between IMs regarding disease severity were explored.

2. Materials and Methods

2.1. SARS-CoV-2 positive samples and healthy controls

Residual EDTA plasma samples (n = 395) from 289 unique SARS-CoV-2 positive (qualitative real-time RT-PCR) patients in whom the IL-6 assay (Mayo Clinic Laboratories, Rochester, MN) was ordered for clinical management were included in the study. Samples were collected in EDTA tubes, placed immediately on wet ice, centrifuged within 2 h of collection (1500 × g for 10 min), aliquoted, and frozen within 30 min (-20 or −80 °C). Samples underwent at least one freeze/thaw cycle prior to testing. Patients were included if they were hospitalized and the sample was collected before the initiation of medications that could potentially alter IM expression, such as monoclonal antibody treatments, convalescent plasma, or antiviral treatments. Additionally, patients were excluded if they were already hospitalized for other complex medical conditions, where SARS-CoV-2 was found incidentally. Patient groups were categorized based on the length of hospital stay as a measure of disease severity. Chart review was performed on each hospitalized patient to record age, sex, relevant clinical history, medications, and SARS-CoV-2 molecular status at the time of collection. Admission and discharge dates were obtained to calculate the length of stay (LOS). The date of symptom onset and the date of sample collection were recorded to calculate the number of days from symptom onset (DFSO) to sample collection. The need for invasive mechanical ventilation, and whether the patient was in the ICU was also recorded. Laboratory results for CRP (serum), ferritin (serum), and D-dimer (plasma) within 24 h of cytokine measurement were recorded, if available.

EDTA plasma samples from symptomatic SARS-CoV-2 (n = 37) positive individuals who did not require hospitalization (non-hospitalized, NH) for COVID-19 symptoms were collected within 72 h after a confirmed positive SARS-CoV-2 PCR result (qualitative real-time RT-PCR, Mayo Clinic, Rochester MN) and then serially collected every 2 days. Sample collection and processing were done in the same manner as the hospitalized patients. Samples were kept frozen (-20 or −80 °C) until testing on the multiplex panel. For the NH patients age, sex, SARS-CoV-2 molecular status, and date of symptom onset were recorded. Additionally, charts were reviewed for at least 2 weeks following collection to ensure that no COVID-19 related hospitalization occurred. Two patients that were initially classified as NH were reclassified into the hospitalized < 10 d group after follow-up review showed that both were admitted to the hospital with COVID-19 pneumonia 5 and 6 days after initial sample collection.

EDTA plasma (n = 119) from healthy donors (median age, IQR = 41.0[33.0,55.0], 48% male) was obtained through the Mayo Clinic Department of Laboratory Medicine and Pathology Biospecimen Donor Program. All samples were kept frozen (-20 or −80 °C) prior to testing. The samples were collected in the same manner as described above for the SARS-CoV-2 positive patients. Exclusion criteria included medications (antibiotic, antiviral, immunosuppressive, etc.), and relevant medical conditions such as autoimmune diseases, infectious diseases including COVID-19, pregnancy, malignancy, and other disorders of circulatory, digestive, respiratory, and endocrine systems, kidney, liver, skin, and thyroid.

All studies were reviewed and approved by the Mayo Clinic Institutional Review Board.

2.2. Custom multiplex assay for immune mediators

A custom human 14-plex assay on the Luminex® FLEXMAP 3D (R&D Systems, Minneapolis, MN, cat#CUST0I704) was used for measurements of MCP-1, MIP-1α, sIL-2Rα, GM-CSF, IFNα, IFNβ, IFN-γ, IL-1β, IL-6, sIL-6Rα, VEG-F, IL-10, IL-18, and TNF. Testing was performed per the manufacturer's instructions. The following analytical parameters were validated prior to performing the study: imprecision, limits of detection and quantification, analytical measurement range (AMR), 97.5th percentile reference interval, accuracy, and analytical specificity (Table 1). No significant cross-reactivity between analytes on the panel was observed (data not shown).

2.3. Exploratory analyses

To determine the prognostic value of the multiplex assay, the earliest available sample collection after a positive SARS-CoV-2 result was included in the analysis. Sample collections took place between 0 and 12 days from symptom onset. For comparison of plasma IM concentrations, the sample cohort was divided into the following groups: healthy controls (HC; n = 119), non-hospitalized (NH; n = 37), hospitalized < 10 days (<10 d; n = 117), hospitalized ≥ 10 days (10 + d; n = 57), and deceased (DEC; n = 16).

Initially, the IM concentrations represented as multiples of the reference median were plotted and analyzed using the Plot by Multiple Conditions interpretive tool in Collaborative Laboratory Integrated Reports (CLIR) web-based multivariate pattern recognition software (https://clir.mayo.edu, 16 Jul 2021) as previously described [14]. Plot by Multiple Conditions in CLIR was also used to explore combinations of IM ratios as potential markers. If the analyte concentration was below the assay's LLOQ or LOD, raw data was used to discern the approximate magnitude of the difference from healthy controls. If the analyte was undetectable, the midpoint between the lowest extrapolated concentration and zero was used in place of the missing value. Clinical significance for this analysis was defined as the group median being above the 97.5th percentile of the reference population (HC group).

Receiver operating characteristic (ROC) curves for 7 of the 14 analytes, excluding those analytes whose median analyte concentrations were below the established LLOQ for each assay, were constructed to evaluate each analyte's ability to discriminate hospitalized from non-hospitalized COV SARS-CoV-2 positive patients. ROC curves were used to determine which analytes best discriminated between those with a short (<10 days) versus long hospital stay (10 + days) and which

Table 1
Analytical performance characteristics of the multiplex assay.

	TNF	***IL-6***	***IFNβ***	***IL-10***	***MCP-1***	***VEGF***	***sIL-6Rα***
AMR (pg/mL)	10.0–900	5.0–315	20.0–1200	7.0–750	40.0–9000	30.0–3462	246–35882
LOD (pg/mL)	4.5	2.9	8.5	3.7	30.8	11.9	47.6
LLOQ (pg/mL)	10.0	5.0	20.0	7.0	40.0	30.0	246.0
Reference Interval (pg/mL)	<10.0	<5.0	<20.0	<7.0	≤245	≤83.4	≤45826
% Spike recovery	85%	74%	85%	104%	138%	94%	88%
	IL-1β	***IFNγ***	***MIP-1α***	***GM-CSF***	***sIL-2Rα***	***IFNα***	***IL-18***
AMR (pg/mL)	20.0–3500	60.0–8500	220–5000	15.0–780	40.0–4000	20.0–1500	65.0–11000
LOD (pg/mL)	10.7	33.8	121	7.8	38.3	8.4	29.9
LLOQ (pg/mL)	20.0	60.0	220	15.0	40.0	20.0	60.0
Reference Interval (pg/mL)	<20.0	<60.0	<220	<15.0	≤1016	<20.0	≤524
% Spike recovery	91%	126%	116%	77%	98%	100%	81%

*Note: For some analytes, the validation of AMR (Analytical Measuring Range) did not include the entire calibrator range. For example, for IL-6, the AMR was only verified up to 315 pg/mL, but the highest calibrator extends to 980 pg/mL.

analytes were able to distinguish patients who were hospitalized versus those who died.

Correlation scatterplots were also constructed for 7 of the 14 analytes. Pair-wise correlation matrices with histograms were constructed for the SARS-CoV-2 positive patients overall and for the following groups: NH, <10 d, 10 + d, and DEC to identify any relationships that may exist between analytes across all SARS-CoV-2 positive patients and within each severity group individually.

2.4. Analysis of candidate immune mediators

To determine whether the candidate IMs selected in the exploratory analysis had practical clinical utility in the setting of COVID-19, univariate group analysis was performed on seven IM assays. These IMs were selected if they displayed adequate analytical sensitivity and clinically significant differences in at least one severity group. For this analysis, results that fell below the LLOQ were assigned a value just below the LLOQ (subtract one from the smallest significant digit). If the value was above the top calibrator, a value just above the highest calibrator was used (add one to the smallest significant digit of the highest calibrator). Medians, IQR, and count (%) above the reference data 97.5th percentile were calculated. Wilcoxon-Mann-Whitney test (5% significance level) was performed to assess median differences between the following patient groups HC, NH, <10 d, 10 + d, DEC.

2.5. Statistical analyses

Instrument analyte concentrations represented as multiples of the reference medians (MOMs) were plotted (CLIR Plot by Multiple Conditions) using CLIR software version 2.23 (Collaborative Laboratory Integrated Reports, https://clir.mayo.edu, 16 Jul 2021). Analyte median MOMs which exceeded the 97.5th percentile of the reference population (above the green shading in Fig. 1) were considered clinically significant [14].

ROC curves were constructed using Analyse-it for Excel (Microsoft, Redmond, WA). Correlation analysis was performed using Spearman's correlation and Fisher Z transformation using Analyse-it for Excel. P-values obtained from correlation analyses were adjusted for multiple comparisons using the Benjamini and Hochberg protocol [15].

Univariate group analysis was performed using Analyse-it for Excel. For each analyte, median (pg/mL) and IQR (Inter-quartile range) were calculated. The medians were tested between group pairs and P-values were calculated using the non-parametric Wilcoxon-Mann-Whitney test (5% significance level). P-values for this analysis were adjusted using the Benjamini and Hochberg protocol [15].

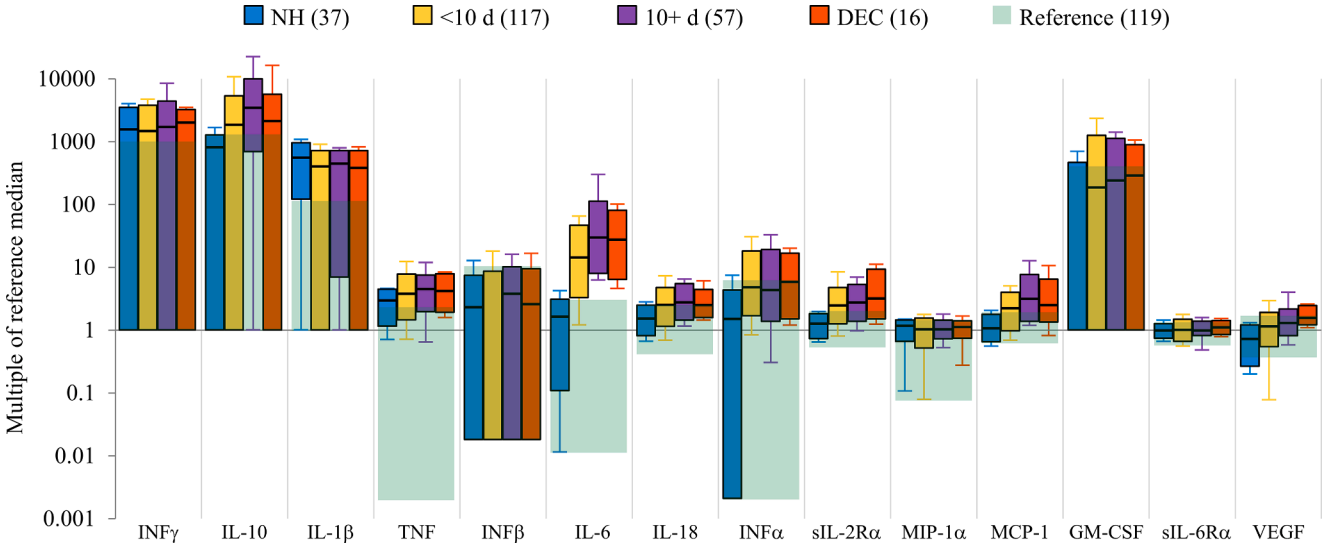

Fig. 1. CLIR plot by multiple conditions of 14 immune mediators' concentrations in SARS-CoV-2 positive patients plotted as multiples of the reference median. Upper whisker end: 97.5th percentile; top of the box: 90th percentile; line in the box: median; bottom of the box: 10th percentile; lower whisker end: 2.5th percentile. Severity groups shown are non-hospitalized (NH), hospitalized < 10 days (<10 d), hospitalized > 10 days (10 + d), and deceased (DEC). If the group median line exceeded the top of the green shaded range (>97.5th percentile of reference population), the analyte differences were considered clinically informative.

3. Results

3.1. Patient cohort characteristics

Table 2 shows the demographic and clinical features of the SARS-COV-2 positive patients. No gender differences were seen between the various disease groups. However, there were significant median age differences between groups, with more severe outcomes observed in older individuals (P=<0.0001–0.0422). Additionally, the percentage of patients in the ICU and on mechanical ventilation increased with length of stay, supporting the use of these parameters as indicators of severity. Where available, CRP, ferritin, and D-dimer concentrations were compared within the cohorts. D-dimer showed significant differences between the DEC and < 10 d (P = 0.0295) and DEC and 10 + d (P = 0.0048) groups, whereas no significant differences were observed between groups for CRP or ferritin.

There were differences between median DFSOs (number of days between symptom onset and sample collection) between the groups. In the non-hospitalized patients, the median [IQR] was 5.0 [4.0, 5.6] days from the onset of symptoms to sample collection, which on average was two days earlier than the hospitalized patients (<10 d, 10 + d). To account for potential differences in IM concentrations due to the timing of sample collection in relation to the symptom onset within the groups, a subset of NH patients (n = 19) in whom samples were collected serially at five and seven days from symptom onset were evaluated. Only one analyte (MCP-1) showed a statistically significant change in the median concentration (149 pg/mL at day 5 versus 136 pg/mL at day 7; P = 0.0027). Given that both values were within the 97.5th reference interval (<or = 245 pg/mL), the changes were not considered to be clinically significant, and the first collection was used in the analysis of all IMs.

3.2. Differences in IM concentrations and association with hospital course

IMs plotted as multiples of the reference median are displayed in Fig. 1. Of the analytes evaluated, IL-6 displayed the best separation between the non-hospitalized and hospitalized groups (<10 d, 10 + d), with the hospitalized patients having minimal overlap with the reference group and non-hospitalized group. Various analytes including MCP-1 (hospitalized and deceased), sIL-2Rα (hospitalized and deceased), IL-18 (hospitalized and deceased), IL-10 (hospitalized and deceased), and TNF (all groups) showed median elevations above the 97.5th percentile of the reference population in some patients, however, there was a less obvious separation between the disease severity groups. Two additional analytes, INF'γ and IL-1β displayed clinically significant elevations in all groups, however, actual median concentrations for all severity groups were lower than the assay's LLOQ, rendering these observations inconclusive. One important observation was that most analytes (11/14) showed the non-hospitalized cohort completely overlapping with the reference population, despite having confirmed SARS-CoV-2 infection. Some analytes (VEGF, IL-10, TNF, IL-18, IFNα) appeared to exhibit differences between severity groups, however, the hospitalized and deceased groups still greatly overlapped with the reference group. sIL-6Rα, MIP-1α, GM-CSF, and INFβ, on the other hand, showed little difference between groups and displayed large overlap with the reference group. Various ratios of IMs were evaluated, and none were superior to IL-6 in differentiating the severity groups (data not shown).

Seven IM candidates were selected for further analysis. Table 3 shows the median, IQR, and % of patients with a result above 97.5th percentile of reference values for sIL-2Rα, IL-6, IL-10 IL-18, TNF, VEGF, and MCP-1. Three analytes (IL-6, IL-10, and MCP-1) showed significant median differences between NH and hospitalized as well as significant differences between the two hospitalized groups with shorter (<10 d) and longer (10 + d) stays. IL-6 median concentrations increased with the severity of the disease. All 10 + d hospitalized patients, as well as deceased patients, had IL-6 concentrations above the 97.5th percentile of the reference value compared to 91% of < 10 d hospitalized patients having an elevated IL-6 and only 14% of outpatients. IL-6 concentrations plotted as a function of the number of symptom days, demonstrated a marked separation between hospitalized patients with non-hospitalized patients and healthy controls. In hospitalized patients, IL-6 showed a weak positive correlation with DFSO (ρ = 0.20, P = 0.0047) over the 12 day time window (Fig. 2). In 94% (179/190) of hospitalized patients, IL-6 was elevated (>5 pg/mL), suggesting that in the first 12 days of infection the timing of IL-6 measurement might not be as critical for predicting patient outcomes (Fig. 2). IL-10 median concentrations were elevated in 32% to 56% of hospitalized patients while no outpatients showed an IL-10 above the 97.5th percentile of the reference population. MCP-1 median concentrations were elevated in 66 to 83% of hospitalized or deceased patients compared to only 5% of NH patients.

The rest of the analytes only had significant median differences between the NH and hospitalized/deceased, but no significant differences between the two hospitalized groups with shorter (<10 d) and longer (10 + d) stays. sIL-2Rα median concentrations were elevated in 62% to 81% of hospitalized or deceased patients while no NH showed an elevated sIL-2Rα. IL-18 median concentrations were elevated in 54% to 56% of hospitalized or deceased patients while 8% of NH showed an elevated IL-18. TNF median concentrations were elevated in 58% to 74% of hospitalized or deceased patients while 32% of NH showed an

Table 2
Cohort characteristics.

	Non-Hospitalized	*Hospitalized < 10 days*	*Hospitalized 10 + days*	*Deceased*
N (Total = 346)	37	117	57	16
Age, Median [IQR]	33.0 [25.6,42.6]	56.1 [42.0,70.6]	60.2 [50.5,74.5]	75.3 [67.1,82.7]
Male (%)	41%	57%	44%	69%
DFSO, Median [IQR]	5.0 [4.0,5.6]	6.9 [3.7,8.3]	7.6 [5.1,9.0]	3.6 [2.6,9.0]
LOS, Median [IQR]	*N/A*	5.0 [3.0,7.0]	14.0 [11.0,24.0]	21.5 [14.4,29.6]
ICU (%)	*N/A*	21%	60%	75%
Mechanical Ventilation during stay (%)	*N/A*	1%	25%	75%
CRP, serum mg/L		72.2 [20.7,134], N = 87	76.6 [57.1,131], N = 40	81.7 [51.5,116], N = 15
Ferritin, serum µg/L		460 [184,1043], N = 42	776 [398,1007], N = 22	278 [144,1069], N = 10
D-Dimer, plasma ng/mL		651 [428,1078], N = 95	720 [527,1183], N = 45	1860 [663,7224], N = 15

*DFSO is the number of days between symptoms onset and sample collection.
*LOS is the length of hospital stay
There were significant differences between patient groups regarding median age, for hospitalized vs. non-hospitalized (Wilcoxon-Mann-Whitney, 5% significance, P-value=<0.0001). Smaller yet significant differences were observed between DEC and 10 + d (P = 0.0051), healthy controls vs non-hospitalized (P = 0.0028), and 10 + d vs < 10 d(P = 0.0422).
Also, significant median D-dimer differences exist between DEC and < 10 d (P = 0.0295) and DEC and 10 + d (P = 0.0048).

Table 3
Median concentrations (pg/mL) of immune mediators across severity groups.

	Non-hospitalized		<10 d hospitalized		10 + d hospitalized		Deceased		P-value				
Analyte	*Median [IQR]*	*^Positive (%)*	*Median [IQR]*	*^Positive (%)*	*Median [IQR]*	*^Positive (%)*	*Median [IQR]*	*^Positive (%)*	*HC with NH*	*NH with < 10 d, 10 + d, DEC*	*< 10 d with 10 + d*	*< 10 d,10 + d with deceased*
sIL-2Rα	644 [516,774]	0 (0%)	1258 [849,1760]	73(62%)	1423 [910,2181]	38 (67%)	1635 [1139,2311]	13 (81%)	**	****, ****, ****	ns	ns, ns
IL-6	4.9 [4.9,4.9]	5 (14%)	24.3 [12.2,49.1]	106(91%)	50.8 [24.2,88.0]	57 (100%)	46.0 [29.7,83.9]	16 (100%)	*	****, ****, ****	***	*, ns
IL-10	6.9 [6.9,6.9]	0 (0%)	6.9 [6.9,8.6]	37(32%)	7.9 [6.9,16.0]	32 (56%)	6.9 [6.9,8.4]	6 (38%)	ns	***, ****, ****	***	ns, ns
IL-18	327 [244,432]	3 (8%)	554 [351,769]	63(54%)	581 [385,753]	31 (54%)	531 [370,677]	9 (56%)	***	****, ****, ***	ns	ns, ns
TNF	9.9 [9.9,9.9]	12 (32%)	11.4 [9.9,15.6]	68(58%)	13.2 [9.9, 17.7]	42 (74%)	12.4 [9.9,13.8]	10 (63%)	***	***, ****, **	ns	ns, ns
VEGF	36.3 [39.8,62.3]	0 (0%)	56.1 [42.0,73.3]	19(16%)	63.1 [46.8,85.8]	15 (26%)	77.7 [60.5,97.8]	5 (31%)	***	***, ****, ****	ns	**, ns
MCP-1	143.6 [118,177]	2 (5%)	305 [193,398]	77(66%)	412 [291,569]	47 (83%)	331 [220,580]	10 (62.5%)	ns	****, ****, ****	***	ns, ns

Note: For this analysis, numbers less than the lower limit of quantitation (LLOQ) are assigned a value just below the LLOQ (subtract one from the smallest significant digit). If the value was above the top calibrator a value just above the highest calibrator was used (add one to the smallest significant digit of the highest calibrator). Corrected P-values (Benjamini Hochberg) were calculated between groups with the Wilcoxon-Mann Whitney test (5% significance level).
^ Positive (%) = number (%) of patients who exceeded the 97.5th percentile of the reference population (Healthy Controls, n = 119)
ns P > 0.05 (not significant),
* P ≤ 0.05.
** P ≤ 0.01.
*** P ≤ 0.001.
**** P ≤ 0.0001.

elevation. VEGF median concentrations were elevated in 16% to 31% of hospitalized or deceased patients while no NH showed a VEGF elevation.

ROC analysis was performed on the seven candidate IMs (IL-6, IL-10 IL-18, sIL-2Rα, MCP-1, VEGF, and TNF) (Fig. 3). IL-6 was the best predictor of hospitalization with a ROC area under the curve (AUC) of 0.96. Two cytokines, sIL-2Rα (AUC 0.87), and MCP-1(AUC 0.88) showed acceptable discrimination between hospitalized and non-hospitalized patients but were not superior to IL-6. Numerous cytokine combinations and cytokine ratios were evaluated but none were as effective at distinguishing outpatients from hospitalized patients as IL-6 alone (data not shown). The ability of the analytes to discriminate between a shorter (<10 d) and longer LOS (10 + d) was also evaluated. The top three candidates to discriminate for the length of stay were IL-6 (AUC 0.70, 95% CI: 0.62–0.87), MCP-1(AUC 0.69, 95% CI: 0.60–0.78), and IL-10 (AUC 0.66, 95% CI: 0.57–0.75), although they demonstrated considerably less discrimination regarding the length of stay as compared to need for hospitalization. Regarding death, only VEGF was able to mildly discriminate between deceased and hospitalized patients (AUC = 0.71, 95% CI: 0.59–0.82), however large overlap with the control group was evident for both groups (ROC curves not shown).

Overall, the IL-6 assay showed superior discrimination and likely has the best predictive power to determine the need for hospitalization based on an early time point measurement. Interestingly, two of the individuals originally recruited into the non-hospitalized cohort had an elevated IL-6 at the time of sample collection and were subsequently hospitalized due to COVID-19 pneumonia. Patient 1 was a 57 y/o old male with hypertension and type 2 diabetes. Symptom onset (day 1) included cough, headache, nausea, and shortness of breath (SOB). On day 3 after symptom onset, the patient tested positive for SARS-COV-2. On days 5 and 8, plasma was collected in an outpatient setting (IL-6 = 16.4 pg/mL and 15.7 pg/mL, respectively). On day 11 the patient presented to the ED with worsening symptoms and was found to be hypoxic, with patchy opacity in the left lung. He was admitted to the hospital. The overall length of stay was 4 days. In comparison to the other non-hospitalized patients, this patient's initial measurement of IL-6 was noticeably elevated 6 days prior to hospitalization. Patient 2 was a 41 y/o male with no significant medical history. SARS-COV-2 PCR was positive on symptom day 3. Initial IL-6 measurement on symptom day 4 was elevated (IL-6 = 14.1 pg/mL). Two days later (day 6) the patient arrived at the ED and was SOB, tachypneic and lymphocytopenic. The patient

Fig. 2. Initial IL-6 concentrations (pg/mL) for all patients versus symptom day. Deceased patients (DEC), hospitalized non-deceased patients (H), non-hospitalized patients (NH), and healthy controls (HC) are shown. The maximum for HCs is denoted by the line. The two non-hospitalized patients reclassified from non-hospitalized to hospitalized (NH to H) are color-coded separately. Spearman's ρ for all hospitalized patients was 0.20 (P = 0.0047), showing a weak positive correlation.

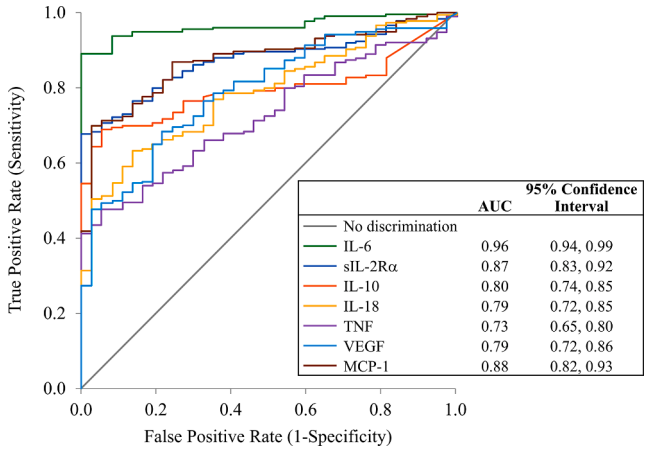

Fig. 3. ROC curve for seven IMs to discriminate between the non-hospitalized (NH) group and the combined non-deceased hospitalized groups (<10 d + 10 + d).

left of his own accord only to return 3 days later (day 9) with worsening symptoms related to COVID-19 pneumonia. The patient had an uncomplicated hospital course and was discharged on day 11. In both patients, the IL-6 concentration at symptom day 4–5 were elevated prior to hospitalization, suggesting that IL-6 may have value as a predictor of the need for hospitalization in SARS-COV-2 infection.

3.3. Correlation analysis for associations between IMs

While some analytes clearly demonstrated more clinical utility than others, closer examination of the relationships between cytokines in the setting of COVID-19 might allow for further mechanistic insight into the disease. Spearman's correlation coefficients between candidate IMs are shown in Fig. 4. When hospitalized patients were examined, all analyte pairs except IL-18 with MCP-1 and sIL-2Rα with MCP-1 show a significant correlation. The strongest correlation in the hospitalized cohort was IL-18 with sIL-2Rα (Spearman's ρ = 0.48, P=<0.0001). In contrast, the non-hospitalized cohort did not show any significant correlations. When combining NH and hospitalized cohorts, all pairs showed significant correlation and the strongest pair was MCP-1 with IL-6 (Spearman's ρ = 0.59, P=<0.0001). Scatter plots and histograms for all possible pairs for 7 candidate analytes are viewable in Figure Supplementary figure 1.

4. Discussion

The measurement of IMs at the earliest available time-point after a positive SARS-CoV-2 PCR test allowed for the evaluation of potential markers that could predict hospitalization and/or length of stay due to COVID-19. Furthermore, marked differences were identified between the IM profiles of non-hospitalized versus hospitalized SARS-COV-2 positive patients. Of the candidate markers chosen for further analysis, significant differences existed between the NH group and all 3 hospitalized groups (<10 d, 10 + d, and DEC) for IL-6, MCP-1, sIL-2Rα, VEGF, IL-18, and TNF. Only IL-6, MCP-1, and IL-10 showed significant differences between groups with a shorter and longer hospital stay (<10 d, 10 + d). When evaluating the clinical performance of each marker in predicting the need for hospitalization, IL-6 was most effective (AUC 0.96). Our findings regarding IL-6 elevations in patients with severe COVID-19 confirm prior reports [16,17,18,19,20,21].

Our study expands on previous findings by showing that the expression of IL-6 in individuals not requiring hospitalization is significantly lower than those individuals who were hospitalized. A study of 471 hospitalized patients and 39 outpatients with mild disease reported that elevations of IL-6, CXCL-10, and GM-CSF were associated with disease severity and accompanied by elevated markers of endothelial injury and thrombosis [5]. A smaller study comparing the distribution of IMs in 37 age- and sex-matched symptomatic and asymptomatic individuals reported that asymptomatic individuals exhibited lower levels of 18 pro and anti-inflammatory cytokines which included IL-6, IFNγ, IP-10, IL-6, GM-CSF, and EN-RAGE/S100A12 [6]. Our study also showed that in the hospitalized groups, IL-6 concentrations showed a stepwise increase with worsening outcomes. When comparing IL-6 to the other evaluated markers, IL-6 was the best predictor of hospitalization when measured early in the time course of the disease. Two individuals recruited as outpatients and later hospitalized had an IL-6 concentration roughly 3 times higher than the upper limit of normal within two days of their positive PCR test. In addition, 7 of 8 (88%) hospitalized patients in whom the IMs were measured within 24 h of their initial symptoms, IL-6

Hospitalized <10 d and 10+ d (n=174)

Analyte	sIL-2Rα	IL-6	IL-10	IL-18	TNF	VEGF	MCP-1
sIL-2Rα		0.26	0.31	0.48	0.46	0.18	0.15
IL-6			0.35	0.21	0.25	0.25	0.41
IL-10				0.35	0.38	0.27	0.32
IL-18					0.28	0.21	0.14
TNF						0.26	0.25
VEGF							0.26
MCP-1							

Non-Hospitalized (n=37)

Analyte	sIL-2Rα	IL-6	IL-10	IL-18	TNF	VEGF	MCP-1
sIL-2Rα		-0.09	0.18	0.35	0.30	0.36	0.32
IL-6			-0.20	0.45	-0.14	-0.10	0.06
IL-10				0.16	0.31	0.41	0.27
IL-18					0.13	0.22	0.31
TNF						-0.06	0.39
VEGF							0.24
MCP-1							

Hospitalized and Non-Hospitalized (n=211)

Analyte	sIL-2Rα	IL-6	IL-10	IL-18	TNF	VEGF	MCP-1
sIL-2Rα		0.49	0.46	0.57	0.52	0.34	0.34
IL-6			0.50	0.41	0.36	0.41	0.59
IL-10				0.44	0.47	0.39	0.43
IL-18					0.35	0.34	0.30
TNF						0.33	0.34
VEGF							0.40
MCP-1							

Spearman's rho
-1 0 1

Fig. 4. Association between IMs. Correlation matrices were constructed for each analyte for hospitalized, non-hospitalized, and combined cohorts. Spearman's rho (ρ) is shown for each pair and statistically significant pairs are highlighted red (P=<0.05).

was elevated at least 2 times the upper limit of normal, and these individuals were all hospitalized for 4 or more days. The individual who had an IL-6 value within the reference interval was hospitalized for only one day. While further studies encompassing a larger number of patients are necessary to confirm these observations, one could speculate that measuring IL-6 shortly after a positive SARS-COV-2 test could help triage patients into low and high-risk categories for complications leading to hospitalization. Furthermore, patients placed into a high-risk category due to elevated IL-6 concentrations might benefit from prophylactic treatment to prevent the need for hospitalization. Prior studies have also demonstrated the effectiveness of IL-6 as a prognostic marker of disease severity. For example, among elderly residents of long-term care facilities, IL-6 showed 100% sensitivity and 90% specificity to predict the development of hypoxemia requiring hospitalization [22].

The significant differences observed for IL-6 and MCP-1 between the < 10 d and $10 +$ d hospitalization groups suggest an important role of these two cytokines in disease severity. IL-6 is a pleiotropic cytokine in circulation which functions to induce synthesis of acute-phase proteins, has effects on antibody production and T-cell development, and can promote differentiation and proliferation of non-immune cells [23]. MCP-1 (CCL-2) is a key chemokine that regulates the migration and infiltration of monocytes/macrophages [24]. Others have reported that elevations in IL-6 and MCP-1 analytes are associated with respiratory failure [25]. A significant correlation was observed between this pair in hospitalized patients, suggesting that these two IMs may synergize to promote disease severity.

When evaluating the correlation between the IMs, some mechanistic insight into COVID-19 pathogenesis might be postulated. For example, a significant correlation was observed between sIL-2Rα and IL-18 in the SARS-CoV-2 positive cohort (NH and hospitalized). IL-18 promotes Th1 cell activation, induces IFNγ, and enhances the cytotoxic activity of CD8 + T cells and NK cells. Interestingly, IL-18 acts as a co-stimulant for Th1 cells to augment IL-2, GM-CSF, and IL-2Rα production [26]. In our study, sIL-2Rα showed significant elevation in the hospitalized groups as compared with the outpatients. sIL-2Rα has been suggested by some to contribute to the lymphopenia in SARS-CoV-2 positive patients. *In vitro* recombinant CD25 (soluble IL-2Rα) has been shown to inhibit T cell proliferation in PBMC, with subsequent rescue by IL-2, suggesting the importance of this signaling pathway for the regulation of proliferation and a potential mechanism of lymphopenia in COVID-19 [27].

A strength of our study is that the assays used were validated to establish analytical performance characteristics and ensure the robustness and accuracy of the results. IMs in which the assay was not considered accurate were eliminated from the more targeted analysis. Using this approach, it is possible to translate our findings into clinical practice and patient management. This approach might also result in the elimination of IMs where the assays were not robust for interpretation. For example, while interferons (α, β, γ) concentrations were detectable, most results were below our established LLOQ which were not considered suitable for clinical interpretation of these analytes in the context of COVID-19. A limitation of the study is that not all samples were collected at the same time point in relation to the onset of symptoms. Statistical analysis comparing the various groups and the time of sample collection did not indicate that this introduced a bias into the study or data analysis. However, a prospective study with samples collected on the same day from symptom onset would help to address whether the timing of IL-6 increase during viral infection affects the patient's outcome. Overall, the differences between IM concentrations in the SARS-CoV-2 positive outpatients versus the hospitalized cohorts observed in this study support that cytokine-mediated dysregulation contributes to disease complication and the need for medical intervention. Of the IMs studied, IL-6 was the best predictive marker for the need for hospitalization and may single-handedly have clinical utility in the early screening of SARS-COV-2 positive patients.

CRediT authorship contribution statement

S. Ashrafzadeh-Kian: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **M.R. Campbell:** Methodology, Validation, Investigation, Writing – review & editing. **J.C. Jara Aguirre:** Data curation. **J. Walsh:** Formal analysis, Software. **A. Kumanovics:** Conceptualization, Investigation. **G. Jenkinson:** Formal analysis, Software. **P. Rinaldo:** Investigation, Formal analysis, Software. **M.R. Snyder:** Conceptualization, Investigation. **A. Algeciras-Schimnich:** Conceptualization, Validation, Investigation, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data is available from the corresponding author upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2021.155790.

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