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ORIGINAL RESEARCH

Unveiling COVID-19 Secrets: Harnessing Cytokines as Powerful Biomarkers for Diagnosis and Predicting Severity

Blanka Wolszczak-Biedrzycka[1], Justyna Dorf[2], Marzena Wojewódzka-Żelezniakowicz[3], Małgorzata Żendzian-Piotrowska[4], Violetta Joanna Dymicka-Piekarska[2], Joanna Matowicka-Karna[2], Mateusz Maciejczyk[4]

[1]Department of Psychology and Sociology of Health and Public Health, University of Warmia and Mazury in Olsztyn, Olsztyn, 10-900, Poland; [2]Department of Clinical Laboratory Diagnostics, Medical University of Bialystok, Bialystok, 15-089, Poland; [3]Department of Emergency Medicine and Disasters, Medical University of Bialystok, Bialystok, 15-089, Poland; [4]Department of Hygiene, Epidemiology and Ergonomics, Medical University of Bialystok, Bialystok, 15-089, Poland

Correspondence: Blanka Wolszczak-Biedrzycka, Email blanka.wolszczak@uwm.edu.pl

Introduction: In coronavirus disease (COVID-19), inflammation takes center stage, with a cascade of cytokines released, contributing to both inflammation and lung damage. The objective of this study is to identify biomarkers for diagnosing and predicting the severity of COVID-19.

Materials and Methods: Cytokine levels were determined in the serum from venous blood samples collected from 100 patients with COVID-19 and 50 healthy controls. COVID-19 patients classified based on the Modified Early Warning (MEWS) score. Cytokine concentrations were determined with a multiplex ELISA kit (Bio-Plex Pro™ Human Cytokine Screening Panel).

Results: The concentrations of all analyzed cytokines were elevated in the serum of COVID-19 patients relative to the control group, but no significant differences were observed in interleukin-9 (IL-9) and IL-12 p70 levels. In addition, the concentrations of IL-1α, IL-1β, IL-1ra, IL-2Rα, IL-6, IL-12 p40, IL-18, and tumor necrosis factor alpha (TNFα) were significantly higher in symptomatic patients with accompanying pneumonia without respiratory failure (stage 2) than in asymptomatic/mildly symptomatic patients (stage 1).

Conclusion: The study revealed that IL-1ra, IL-2Rα, IL-6, IL-8, IL-12 p40, IL-16, and IL-18 levels serve as potential diagnostic biomarkers in COVID-19 patients. Furthermore, elevated IL-1α levels proved to be valuable in assessing the severity of COVID-19.

Keywords: cytokines, COVID-19, MEWS score, biomarkers, severity, diagnosis

Introduction

The pathogenesis of the cytokine storm (CS) in coronavirus disease 2019 (COVID-19), which is observed mainly in patients with severe symptoms of the disease, has attracted considerable research interest.[1] Clinical data indicate that most patients who died from COVID-19 and patients with severe symptoms of the disease did not present with serious clinical symptoms in the early stage of infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 virus).[2] The clinical state of these patients deteriorated unexpectedly in the subsequent stages of disease, and the acute respiratory distress syndrome (ARDS) and the multiple organ dysfunction syndrome (MODS) led to death within a short period of time.[3,4]

When a compromised immune system is unable to fight the infection, the inflammation can trigger hypercytokinemia, commonly referred to as the CS, followed by the cytokine release syndrome (CRS), also known as the cytokine storm syndrome (CSS).[5,6] The CS, in particular the local overproduction of cytokines, is the key determinant of disease severity and mortality in patients infected with the SARS-CoV-2 virus.[7] Numerous studies have shown that ARDS in severe/critical COVID-19 is not directly triggered by the virus' mechanism of action, but occurs in consequence of CS/CRS.[7–9] The spike (S) protein on the surface of the SARS-CoV-2 virus activates alveolar epithelial cells, macrophages, and circulating blood monocytes via Toll-like membrane receptors and stimulates the production of proinflammatory

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cytokines and chemokines which mobilize immune cells, in particular monocytes and T cells, leading to pneumonia.[10] In patients infected with the SARS-CoV-2 virus, an inflammation develops due to an imbalance in the activity of proinflammatory and anti-inflammatory cytokines.[11] Cytokines are proteins synthesized by cells that directly participate in the immune response. Based on their function, cytokines can be divided into the following groups: interleukins (IL), tumor necrosis factors (TNF), transforming growth factors (TGF), colony-stimulating factors (CSF), and chemokines.[12,13] Cytokines have pleiotropic properties because they act on different cell populations and elicit different effects.[14] Cytokines can also enter into synergistic or antagonistic interactions.[15] Cytokines are released in response to viral infections, including infections with the SARS-CoV-2 virus.[16] When the immune system is compromised, the body cannot fight the infection, which leads to CS and, consequently, CRS.[17] The CS increases the severity of disease symptoms and the risk of mortality in patients infected with the SARS-CoV-2.[18,19] According to clinical data, most critically ill COVID-19 patients and patients who died from COVID-19 did not present with serious clinical symptoms in the early stage of infection. Their condition deteriorated rapidly, and they died from ARDS and MODS within a short period of time.[16,20,21]

Proinflammatory cytokines include interleukins (ILs) IL-1α, IL-1β, IL-2Ra, IL-6, IL-8, IL-17, IL-18, and IL-23, as well as the tumor necrosis factors α and β (TNFα and TNFβ).[12,22] The main anti-inflammatory cytokines include the IL-1 receptor antagonist (IL-1ra), IL-4, IL-9, and IL-10.[23]

Research has confirmed that the SARS-CoV-2 virus selectively induces high IL-6 levels and that IL-6 levels are significantly correlated with disease severity.[24] A similar correlation has been reported for IL-8, IL-10, and IL-17.[25,26] High levels of these cytokines, in particular IL-6, are unfavorable prognostic markers because they shorten survival.[27]

Infections caused by the SARS-CoV-2 virus with a similar severity of early symptoms have a high risk of complications and, in extreme cases, can lead to death, especially among the elderly.[28] This study aims to pinpoint specific biomarkers that can serve both as diagnostic indicators and predictors of the severity of COVID-19. The role of various cytokines in the progression of COVID-19 was evaluated by analyzing the activity of proinflammatory and anti-inflammatory cytokines in patients infected with the SARS-CoV-2 virus and experiencing different severity of disease symptoms.

Materials and Methods

Experimental Design

The study was approved by the Bioethics Committee of the Medical University in Białystok (decision No. APK.002.353.2021–28.01.2021 r.). The study was conducted in accordance with the World Medical Association Declaration of Helsinki for ethical principles for medical research involving human subjects. All research participants gave their written consent to participate in the study.

Studied COVID-19 Population

The studied population consisted of non-vaccinated 100 patients with a positive result of a COVID-19 PCR test (nasopharyngeal swab) who were admitted to the Emergency Department of the University Clinical Hospital in Białystok between 20 January and 20 November 2021.

The severity of COVID-19 was assessed based on the Modified Early Warning Score (MEWS) which is recommended by the Polish Society of Epidemiology and Infectious Diseases and relies on the following parameters: systolic blood pressure, heart rate, respiratory rate, body temperature, and neurological symptoms. Four stages of COVID-19 progression were described based on the above parameters: 1) asymptomatic and mildly symptomatic infection, 2) symptomatic infection with pneumonia without symptoms of ARDS, 3) symptomatic infection with pneumonia and symptoms of ARDS, 4) symptomatic infection with MODS (Table S1).

The studied population was divided into two groups. Group 1 consisted of asymptomatic and mildly symptomatic patients (stage 1), whereas group 2 consisted of symptomatic patients with pneumonia without symptoms of ARDS (stage 2) and symptomatic patients with pneumonia and symptoms of ARDS (stage 3). None of the studied patients presented with disease symptoms characteristic of stage 4 based on the MEWS score.[29]

The studied patients were subjected to body mass index (BMI), imaging examinations (radiography and computed tomography of the chest) and laboratory tests, including complete blood count (CBC), coagulation parameters (PT, APTT, D-dimers), kidney function tests (creatinine levels with estimated glomerular filtration rate (eGFR), urea), electrolyte levels (Na^+, K^+), and lactate dehydrogenase (LDH) activity, oxygen saturation and C-reactive protein (CRP). Demographic parameters (sex, age), length of hospital stay (days), comorbidities (present, absent), hematological disorders (present, absent), diabetes (present, absent), hypertension (present, absent), obesity (present, absent), heart disease (present, absent), history of cancer (present, absent), and clinical symptoms, including fever (present, absent), cough (present, absent), dyspnea (present, absent) and ARDS (present, absent), were analyzed.

In both groups, blood for analyses was collected form the basilic vein into clot activator tubes. The serum was separated by centrifugation (1000 × g, 20 minutes), and the samples were stored at a temperature of −80°C until analysis.

Control Group

The control group consisted of 50 healthy subjects without a history of COVID-19 or reported comorbidities.

Cytokine Detection

Interleukins IL-1α, IL-1β, IL-1ra, IL-2Rα, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-16, IL-17, IL-18, TNFα, and TNFβ were identified with the use of the Bio-Plex Pro™ Human Cytokine Screening Panel (Biorad) and the Bio-Plex Multiplex system based on the Luminex xMAP technology. The Bio-Plex technology relies on the ELISA method. Antibodies targeting specific biomarkers form covalent bonds with magnetic beads. In the next stage, magnetic beads react with the samples containing these biomarkers. The sample is rinsed to remove unbound protein, and a biotinylated detection antibody is added to produce a sandwich compound. The final product (complex) is obtained by adding streptavidin phycoerythrin (SA-PE) conjugate. The results are read in the Bio-Plex 200 system. The efficacy of the applied method is comparable to that of a standard ELISA assay.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, La Jolla, USA). The Shapiro–Wilk test was used to determine the normality of distribution.

In the case of the lack of normal distribution, the Mann–Whitney *U*-test was used. The results were presented as median (minimum-maximum), and the value of $p<0.05$ was considered statistically significant. Using the corresponding receiver operating characteristic (ROC) curve approach and computing the area under the curve (AUC), the diagnosis and prediction of the severity indices were evaluated. Youden's index was used to establish the best cutoff values for biomarkers.

Results

Characteristics of the Study Group

A total of 100 COVID-19 patients aged 36 to 87 were analyzed. The studied population consisted of 35% males and 65% females. The patients were divided into two groups based on their MEWS scores (Table S1): stage 1 (asymptomatic/mildly symptomatic) – 53%, and stages 2+3 (pneumonia without/with ARDS) – 47%. Approximately 67% of the patients were hospitalized for less than 10 days, 12% – for 10–20 days, and 21% – for more than 20 days. Comorbidities, mainly hypertension (36%), ischemic heart disease (26%) and diabetes (17%), were reported by 57% of the studied subjects. The most prevalent symptoms were fever and dyspnea which were noted in 37% and 35% of the patients, respectively. The studied population with laboratory test results is presented in Table 1.

Table 1 Characteristics of the Studied Population with Selected Laboratory Test Results

	All Patients with COVID-19	COVID-19 Severity According to MEWS		*p-value*
Clinical parameters		1	2	
Number of patients	100	53 (53%)	47 (47%)	
Age (years)				0.104
≤55	32 (32%)	12 (37%)	20 (63%)	
56–75	31 (31%)	15 (48%)	16 (52%)	
>76	37 (37%)	26 (70%)	11 (30%)	
Sex				0.252
Female	65 (65%)	30 (65%)	35 (75%)	
Male	35 (35%)	23 (35%)	12 (25%)	
Length of hospital stay (days)				0.065
≤10	67 (67%)	36 (53%)	31 (47%)	
10–20	22 (22%)	13 (59%)	8 (41%)	
>20	11 (11%)	4 (54%)	9 (46%)	
BMI				0.322
<19	15 (15%)	12 (80%)	3 (20%)	
19–25	78 (78%)	48 (61%)	30 (39%)	
>25	7 (7%)	5 (71%)	2 (29%)	
Oxygen saturation (%)				0.156
≥95%	21 (21%)	11 (52%)	10 (48%)	
94–90%	64 (64%)	44 (68%)	20 (32%)	
<90%	15 (15%)	5 (33%)	10 (67%)	
Comorbidities (n,%)				0.085
Absent	43 (43%)	16 (37%)	19 (63%)	
Present	57 (57%)	29 (51%)	28 (49%)	
Hypertension	22 (37%)	11 (50%)	11 (50%)	
Diabetes mellitus	11 (11%)	5 (45%)	6 (55%)	
Obesity	2 (2%)	1 (50%)	1 (50%)	
Coronary artery disease	15 (15%)	9 (60%)	6 (40%)	
Other (eg cancers, hematological)	7 (7%)	4 (57%)	3 (43%)	
SymptomsCough				0.258
Absent	15 (15%)	1 (7%)	14 (93%)	
Present	85 (85%)	42 (49%)	43 (51%)	
Fever				0.236
Absent	63 (63%)	16 (25%)	47 (75%)	
Present	37 (37%)	37 (100%)	0 (0%)	
Dyspnea				0.245
Absent	35 (35%)	19 (54%)	16 (46%)	
Present	65 (65%)	34 (52%)	31 (48%)	
Respiratory failure				0.328
Absent	7 (7%)	4 (57%)	3 (43%)	
Present	93 (93%)	49 (52%)	44 (48%)	
Laboratory resultsWBC x10^3/mm^3	6.84 (4.61–8.94)	6.63 (4.61–8.15)	7.09 (5.65–8.94)	0.072
Neutrophils x10^3/mm^3	4.86 (3.02–6.68)	4.68 (3.02–6.68)	5.12 (3.14–6.17)	0.045

(*Continued*)

Table 1 (Continued).

	All Patients with COVID-19	COVID-19 Severity According to MEWS		*p-value*
Lymphocytes $x10^3mm^3$	0.91 (0.41–1.35)	1.15 (0.81–1.35)	0.82 (0.41–1.13)	0.228
Monocytes $x10^3mm^3$	0.58 (0.27–0.67)	0.52 (0.28–0.67)	0.61 (0.27–0.60)	0.196
Eosinophils $x10^3/mm^3$	0.025 (0.00–0.035)	0.034 (0.00–0.035)	0.018 (0.00–0.010)	0.486
Basophils $x10^3/mm^3$	0.017 (0.01–0.020)	0.018 (0.01–0.020)	0.016 (0.01–0.020)	0.157
RBC $x10^6/mm^3$	4.31 (3.97–4.86)	4.42 (4.01–4.86)	4.22 (3.97–4.66)	0.603
PLT $x10^3/mm^3$	217.43 (147.00–266.00)	227.34 (160.50–266.00)	200.68 (147.00–248.00)	0.067
Creatinine mg/dl	0.998 (0.780–1.130)	0.978 (0.785–1.150)	1.002 (0.780–1.130)	0.777
LDH U/l	432.23 (280.00–561.00)	420.26 (280.00–493.00)	458.45 (323.000–561.000)	0.041
INR	1.722 (1.080–1.300)	1.614 (1.080–1.300)	1.796 (1.091–1.280)	0.085
CRP	41.76 (11.67–117.76)	30.23 (11.67–82.34)	52.35 (23.34–117.76)	0.098
Fibrinogen mg/dl	487.34 (322.00–623.00)	457.23 (322.00–605.00)	518.81 (385.00–623.00)	0.146
D-dimers µg/l	3521.34 (590.00–1855.00)	3124.89 (605.50–1639.50)	3897.28 (590.00–1855.00)	0.308

Abbreviations: COVID-19, coronavirus disease 2019; INR, international normalized ratio; LDH, lactate dehydrogenase; PLT, platelets; RBC, red blood cells; WBC, white blood cells.

A Comparison of Proinflammatory and Anti-Inflammatory Cytokine Levels in COVID-19 Patients and the Control Group

The cytokine analysis revealed that the concentrations of all evaluated proteins were higher in COVID-19 patients than in the control group. With the exception of IL-9 and IL-12 p 70, the observed differences were statistically significant ($p<0.05$) (Figure 1 and Table 2).

A Comparison of Proinflammatory and Anti-Inflammatory Cytokine Levels in COVID-19 Patients with Different MEWS Scores

The concentrations of IL-1α, IL-1β, IL-1ra, IL-2Rα, IL-6, IL-12 p40, IL-18, and TNFα were also significantly higher in symptomatic patients with accompanying pneumonia without symptoms of ARDS (group 2) than in asymptomatic or mildly symptomatic patients (group 1) (Figure 2 and Table 3).

A ROC Analysis of Pro- and Anti-Inflammatory Cytokine Levels for Differentiating Between COVID-19 Patients and the Control Group

The ROC analysis revealed that the evaluated cytokines are potentially useful parameters for differentiating between COVID-19 patients and healthy subjects (Table 4). The optimal cut-off values were calculated, and the ROC curves are presented in Figure 3. The area under the curve (AUC) >0.9 calculated for IL-1ra, IL-6, IL-8, IL-12 p40, IL-16 was determined at 0.9633, 0.9864, 0.9642, 0.9304, 0.9468 respectively.

A ROC Analysis of Proinflammatory and Anti-Inflammatory Cytokines for Differentiating COVID-19 Patients with Different MEWS Scores

The ROC analysis demonstrated that the evaluated cytokines are potentially useful biomarkers for differentiating COVID-19 patients with different disease severity (Table 5). The optimal cut-off values were calculated, and the ROC curves are presented in Figure 4. The AUC value >0.9 calculated for AUC IL-1α and were determined at 0.9611.

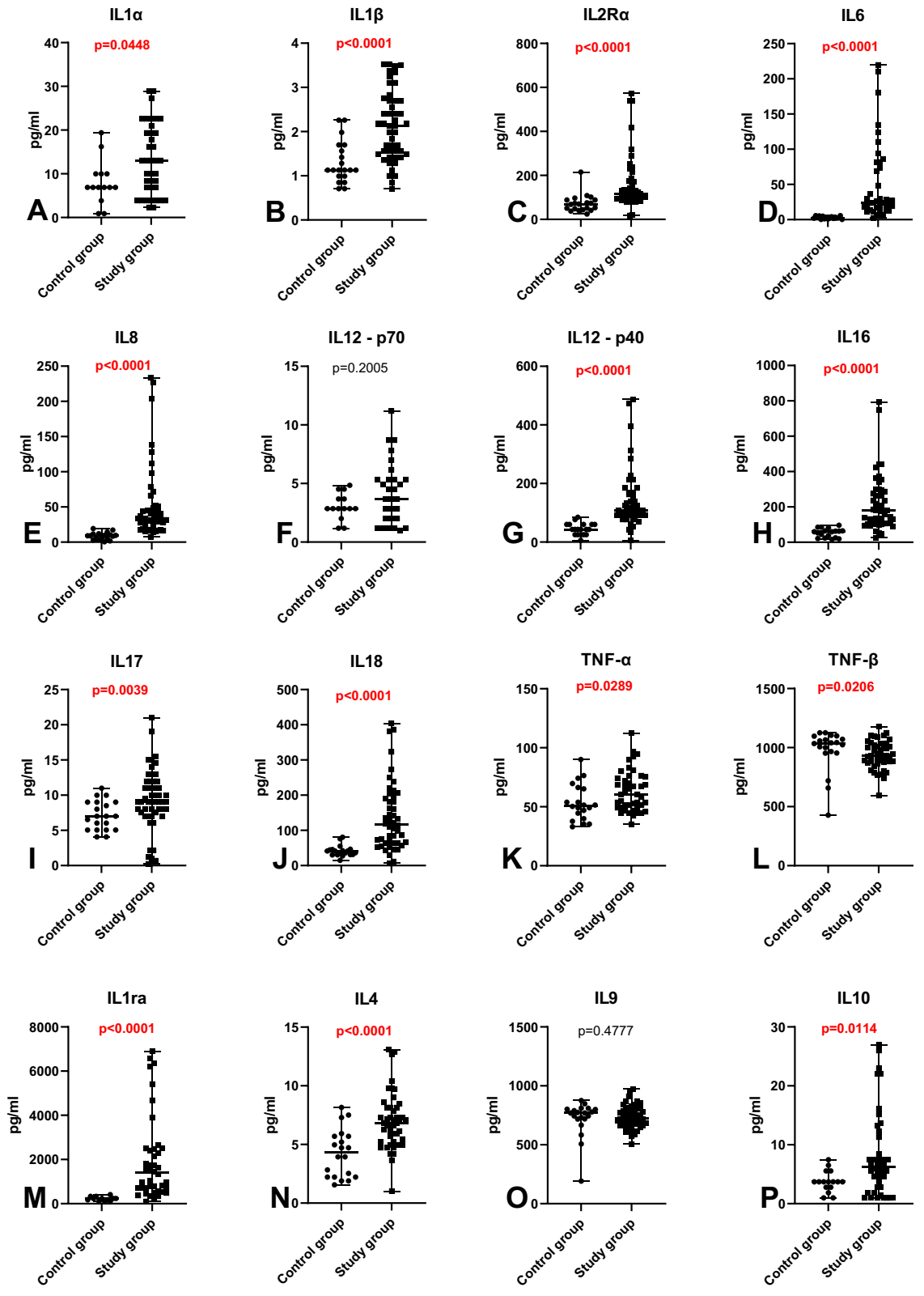

Figure 1 A comparison of proinflammatory (**A**–**L**) and anti-inflammatory (**M**–**P**) cytokine levels in COVID-19 patients and the control group.
Notes: Statistically significant p<0.05 (red), not statistically significant (black).

Table 2 Serum Levels of Proinflammatory and Anti-Inflammatory Cytokines in COVID-19 Patients and the Control Group

Parameter	Control Group Median (min - max) (pg/mL)	COVID-19 Patients Median (Min - Max) (pg/mL)	Significance, *p*
IL-1alpha	6.88 (0.8900–19.37)	13.08 (2.350–28.92)	0.0462
IL-1beta	1.130 (0.7100–2.260)	2.12 (0.7100–3.530	< 0.0001
IL-1ra	236.3 (100.7–409.2)	1398 (100.7–6898)	< 0.0001
IL-2Ralpha	68.85 (24.01–214.8)	114.2 (18.13–572.9)	< 0.0001
IL-4	4.33 (1.550–8.150)	6.84 (1.000–13.08)	< 0.0001
IL-6	2.23 (0.4100–5.690)	24.07 (2.100–219.9)	< 0.0001
IL-8	9.11 (0.6000–19.32)	33.38 (7.630–233.3)	< 0.0001
IL-9	769.2 (191.4–876.1)	726.3 (505.4–972.8)	0.4767
IL-10	3.7 (0.9800–7.450)	6.265 (0.9800–26.95)	0.0157
IL-12 (p70)	2.85 (1.180–4.850)	3.68 (1.000–11.18)	0.2002
IL-12 (p40)	43.34 (5.790–84.92)	108.7 (5.790–487.1)	< 0.0001
IL-16	59.82 (15.88–96.56)	180.1 (25.30–791.7)	< 0.0001
IL-17	7.02 (4.080–10.99)	9 (0.2500–21.02)	0.0045
IL-18	41.4 (14.85–80.52)	116.5 (6.690–404.2)	< 0.0001
TNF-alpha	50.9 (33.06–90.03)	60.6 (35.37–112.5)	0.0388
TNF-beta	1037 (428.4–1126)	931.8 (593.5–1178)	0.0217

Abbreviations: IL, interleukin; TNF, tumor necrosis factor.

Discussion

New biomarkers for effective diagnosis of COVID-19 are needed. Our previous study of inflammatory markers in patients infected with the SARS-CoV-2 virus demonstrated that IL-6 levels increase with the severity of COVID-19 symptoms classified based on the MEWS score.[30] The present study was undertaken to examine the concentrations of other proinflammatory and anti-inflammatory cytokines in COVID-19 patients and to assess, for the first time, the relationship between changes in cytokine levels and the MEWS score. The MEWS score is calculated based on systolic blood pressure, heart rate, respiratory rate, body temperature, and neurological parameters, and it is used to classify patients into four groups/stages of COVID-19 severity. Diagnostic scales are useful tools for assessing disease progression in clinical practice.[31] In the literature, the correlations between selected cytokine levels and the MEWS score have been examined in various diseases, including acute ischemic stroke[21] and cirrhosis,[32] but these parameters have never been assessed in patients infected with the SARS-CoV-2 virus.

In the present study, the concentrations of all cytokines and TNF were significantly higher in patients infected with the SARS-CoV-2 virus than in healthy subjects, which confirms that cytokines are released in COVID-19 patients. Based on their MEWS scores, the studied COVID-19 patients were classified into stage 1 (asymptomatic or mildly symptomatic) or stage 2 (pneumonia without ARDS) of disease progression. Therefore, a significant increase in cytokine levels (mainly IL1-ra, IL-2ra, IL-6, IL-8, IL-12p40, IL-16, IL-18) in the early stage of infection can be an important diagnostic factor, in particular in mildly symptomatic patients, that can be helpful in selecting the appropriate treatment, preventing disease progression and worsening of symptoms. It should also be noted that most of the analyzed cytokines were also highly useful markers for diagnosing COVID-19 (mainly IL-1ra, IL-6, IL-8, IL-16) and differentiating between patients with different disease severity (IL-1α). The AUC for this proteins were >0.9, sensitivity and specificity >90%.

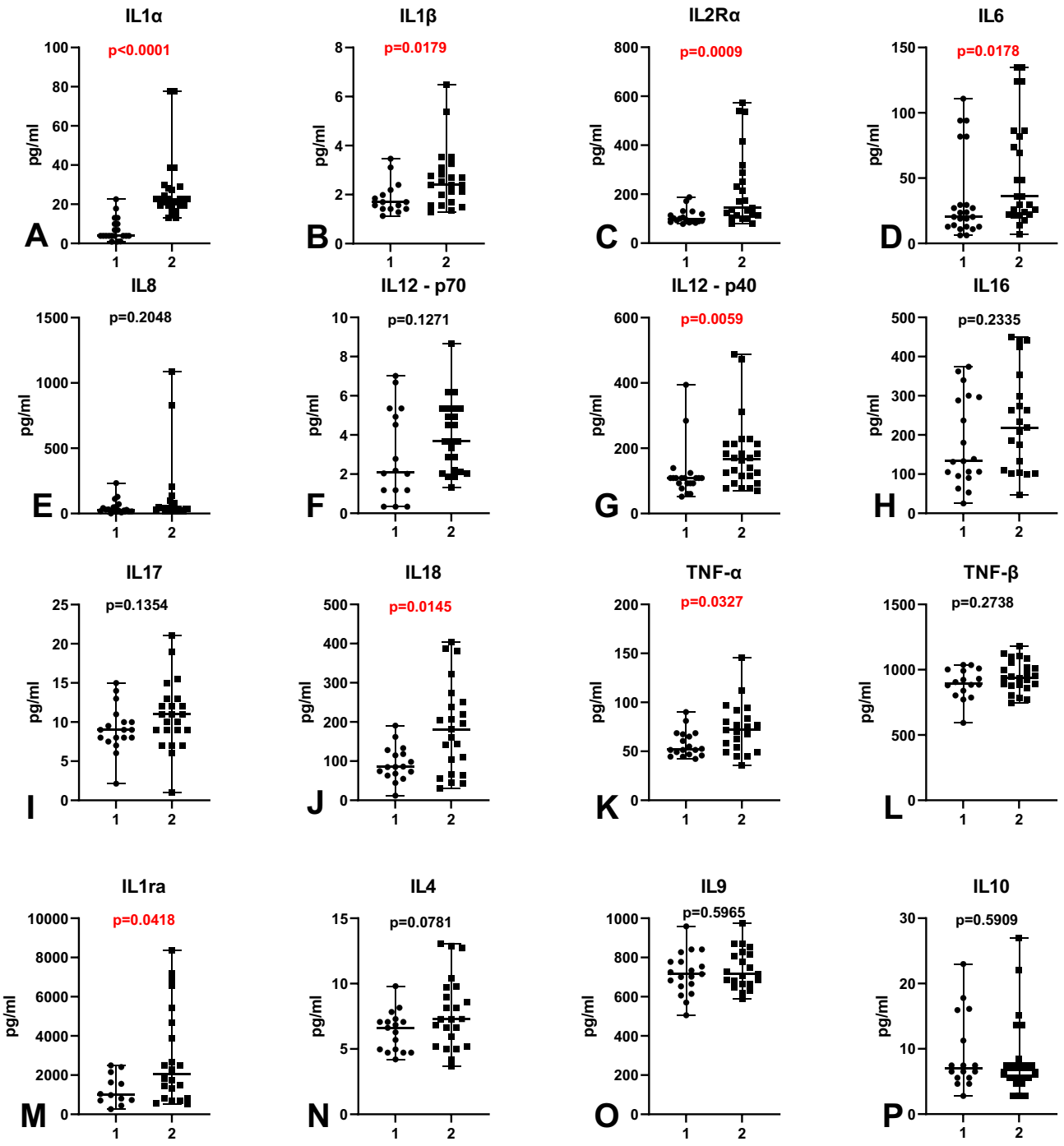

Figure 2 A comparison of proinflammatory (**A**–**L**) and anti-inflammatory (**M**–**P**) cytokine levels in asymptomatic or mildly symptomatic patients (stage 1) and symptomatic patients with accompanying pneumonia without symptoms of the acute respiratory distress syndrome (stage 2).
Notes: Statistically significant p<0.05 (red), not statistically significant (black).

According to research, IL-1, IL-6, and TNF-α play a particularly important role in the initiation of CS in patients infected with the SARS-CoV-2 virus.[33–35] Liu et al[36] analyzed the cytokine profiles of COVID-19 patients and found that cytokine levels were significantly higher in the blood plasma of infected patients than in healthy subjects. They also reported a strong correlation between severe lung damage and elevated concentrations of IFN-γ, IFN-α2, IL-1ra, IL-2, 4, 6, 10, 12 and 17.

Table 3 Serum Levels of Proinflammatory and Anti-Inflammatory Cytokines in Asymptomatic or Mildly Symptomatic Patients (Stage 1) and Symptomatic Patients with Accompanying Pneumonia Without Symptoms of the Acute Respiratory Distress Syndrome (Stage 2)

Parameter	MEWS 1 Median (Min - Max) (pg/mL)	MEWS 2 Median (Min - Max) (pg/mL)	Significance, *p*
IL-1alpha	3.85 (0.8900–22.54)	22.54 (13.08–77.83)	< 0.0001
IL-1beta	1.7 (1.130–3.460)	2.4 (1.280–6.500)	0.0196
IL-1ra	1010 (262.0–2499)	2045 (510.9–8353)	0.0538
IL-2Ralpha	97.87 (78.46–187.4)	144.6 (79.58–572.9)	0.0013
IL-4	6.62 (4.210–9.810)	7.29 (3.670–13.08)	0.0794
IL-6	20.31 (6.180–110.8)	36.21 (6.980–134.7)	0.0191
IL-8	28.81 (1.000–233.3)	38.8 (15.26–1085)	0.2047
IL-9	715.3 (505.4–958.5)	716.2 (588.9–972.8)	0.5973
IL-10	6.975 (2.780–22.98)	6.74 (2.780–26.95)	0.5918
IL-12 (p70)	2.11 (0.3400–7.010)	3.68 (1.340–8.680)	0.1278
IL-12 (p40)	108.7 (51.93–394.0)	165.7 (68.66–487.1)	0.0068
IL-16	133.1 (25.30–373.8)	218.6 (46.43–450.5)	0.2333
IL-17	9.01 (2.140–14.98)	10.99 (1.000–21.02)	0.1359
IL-18	85.31 (11.94–190.6)	181 (30.24–404.2)	0.016
TNF-alpha	52.04 (42.29–90.03)	71.96 (35.37–145.9)	0.0344
TNF-beta	895.4 (593.5–1037)	939.9 (742.8–1178)	0.1718

Abbreviations: IL, interleukin; TNF, tumor necrosis factor.

Table 4 Area Under the Curve (AUC) for the Analyzed Cytokines for Differentiating Between COVID-19 Patients and the Control Group

Parameter	AUC	P-value	Cut-Off	Sensitivity (%)	Specificity (%)	95% Confidence Interval
IL-1alpha	0.6814	0.0465	7.650	68.42	64.29	0.5323 to 0.8305
IL-1beta	0.8109	<0.0001	1.525	72.92	70	0.7038 to 0.9180
IL-1ra	0.9633	<0.0001	351.0	90.48	91.67	0.9155 to 1.000
IL-2Ralpha	0.8602	<0.0001	88.78	81.63	80	0.7586 to 0.9618
IL-4	0.7995	0.0001	5.330	72.34	70	0.6790 to 0.9200
IL-6	0.9864	<0.0001	5.935	97.44	100	0.9588 to 1.000
IL-8	0.9642	<0.0001	15.41	93.33	88.89	0.9219 to 1.000
IL-9	0.556	0.4724	752.5	60.87	55	0.4052 to 0.7067
IL-10	0.7038	0.0158	4.165	71.74	75	0.5746 to 0.8330
IL-12 (p70)	0.6219	0.1995	3.265	58.62	64.29	0.4585 to 0.7854

(*Continued*)

Table 4 (Continued).

Parameter	AUC	P-value	Cut-Off	Sensitivity (%)	Specificity (%)	95% Confidence Interval
IL-12 (p40)	0.9304	<0.0001	64.51	88.89	89.47	0.8682 to 0.9926
IL-16	0.9468	<0.0001	87.96	91.49	94.44	0.8946 to 0.9990
IL-17	0.7168	0.0046	8.260	61.54	65	0.5957 to 0.8380
IL-18	0.8942	<0.0001	49.37	87.5	84.21	0.8151 to 0.9733
TNF-alpha	0.6625	0.0383	53.19	63.64	60	0.5173 to 0.8077
TNF-beta	0.6793	0.0213	1000	67.39	70	0.5264 to 0.8323

Abbreviations: IL, interleukin; TNF, tumor necrosis factor.

Interleukin-1 is a group of cytokines that includes IL-1α, IL-1β, and IL-1ra. These cytokines stimulate the immune system and induce inflammatory processes by activating T cells, eosinophils, basophils, dendritic cells, and natural killer (NK) cells.[35,37] This observation which explains the significant increase in IL-1α, IL-1β, and IL-1ra concentrations in the current study.

The concentration of IL-18 also was also significantly higher in COVID-19 patients than in healthy subjects, and this parameter increased with a rise in the severity of disease symptoms. This cytokine is produced by macrophages, dendritic cells, and epithelial cells, and it induces Th1-, Th2-, and Th17-mediated immune responses, which increases the synthesis of IL-4 and IL-13.[36,38,39] Interleukin-18 can trigger a strong inflammatory response, which suggests that this cytokine may have a pathophysiological function in diseases associated with inflammation, including COVID-19.[40–43] According to many researchers, IL-6 plays a key role in immune system regulation, which is why this cytokine should be regarded as a priority biomarker in SARS-CoV-2 infection.[24,25,27] This cytokine is produced mainly by monocytes and macrophages, and, to a smaller extent, by T and B cells, vascular endothelial cells, keratinocytes, and chondrocytes.[44] Interleukin-6 has pleiotropic activity, and it exerts various effects on innate and adaptive immune system cells.[25] This cytokine activates the differentiation of B cells, stimulates cytotoxic T cells, stimulates the secretion of IL-2 by T cells and the production of acute-phase proteins (APPs) by liver cells.[44–47] Interleukin-6 binds to the transmembrane IL-6 receptor (IL-6R) to produce the IL-6/IL-6R complex,[48] which results in the formation of the gp130 protein homodimer and the activation of the intercellular signal transduction pathway involving TYK-2, JAK1, and JAK2 kinase → transcription factor STAT3 → IL-6 transcription (Chonov et al 2019; Hwang et al 2019; Zheng et al 2021). Research has shown that elevated IL-6 levels can activate the clotting system and increase vascular permeability, which promotes the spread of pro-inflammatory mediators and numerous inflammatory cells.[49,50] In the present study, D-dimer levels were significantly higher in patients with pneumonia without ARDS (stage 2) than in asymptomatic or mildly symptomatic patients (stage 1), which points to intensified coagulation and fibrinolysis in this group of patients. It has been previously investigated that D-dimer is associated with COVID-19 mortality.[51,52] Elevated IL-6 levels are associated with lymphopenia, decreased cytotoxicity of Tc-CD8+ cells, and activation of the vascular endothelium.[53–55] The concentration of IL-6 also increases significantly as a result of inflammatory damage to the lungs.[56] In the present study and in our previous research, IL-6 levels were significantly elevated in patients with SARS-CoV-2 infection.

Tumor necrosis factor α also plays an important role in SARS-CoV-2 infection and CS.[57] The concentration of TNF-α increases at the beginning of infection, remains elevated during disease progression, and increases considerably when the patient's condition deteriorates.[58] This cytokine is produced by macrophages, monocytes, neutrophils, activated lymphocytes, mast cells, astrocytes, adipocytes, and smooth muscle cells. Similarly to IL-6, TNF-α stimulates the secretion of APPs.[59,60] These observations explain the observed increase in TNF-α concentration in COVID-19 patients in the current study.

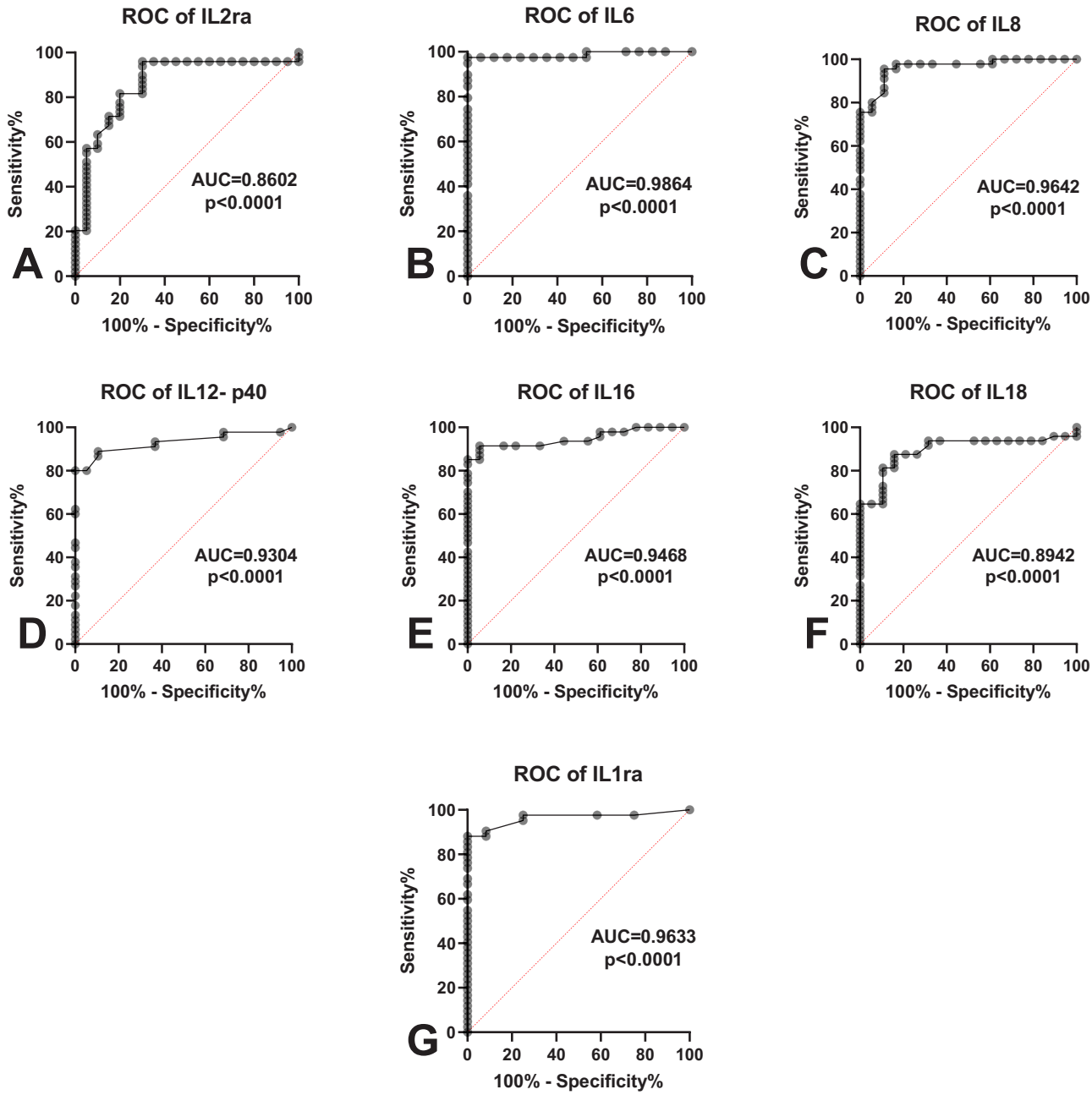

Figure 3 Receiver operating characteristic (ROC) curves of proinflammatory (**A**–**F**) and anti- inflammatory (**G**) cytokines for differentiating between COVID-19 patients and the control group.

The statistical analysis revealed a significant ($p<0.05$) increase in the concentrations of IL-1α, IL-1β, IL-1ra, IL-2Rα, IL-6, IL-12 p40, IL-18, and TNFα with a rise in disease severity classified based on the MEWS score. Diagnostic scales are highly useful in clinical practice because they facilitate the assessment of a patient's condition and the selection of the optimal treatment.[61–64] In Poland, most COVID-19 patients were classified based on the MEWS score[65] because a diagnostic scale specific for this disease has not been developed. Potential biomarkers of significant differences between groups of patients characterized by minor differences in disease symptoms are needed. Significant differences in cytokine levels between asymptomatic/mildly symptomatic patients and patients with moderate symptoms of COVID-19 have not been described in the literature to date. In studies where cytokine levels were analyzed, only high levels of IL-6, TNFα, and IL-2ra were reported in

Table 5 Area Under the Curve (AUC) for the Analyzed Cytokines for Differentiating COVID-19 Patients with Different MEWS Scores

Parameter	AUC	P-value	Cut-off	Sensitivity (%)	Specificity (%)	95% Confidence Interval
IL-1alpha	0.9611	<0.0001	14.08	92.59	90	0.9031 to 1.000
IL-1beta	0.7275	0.0191	2.050	69.57	73.33	0.5617 to 0.8933
IL-1ra	0.7045	0.0517	1472	63.64	58.33	0.5311 to 0.8780
IL-2Ralpha	0.7959	0.0013	113.9	73.91	72.22	0.6570 to 0.9348
IL-4	0.665	0.0776	6.955	56.52	58.82	0.4969 to 0.8331
IL-6	0.6983	0.0186	26.50	60	60.87	0.5471 to 0.8494
IL-8	0.6172	0.2002	33.10	63.64	63.16	0.4421 to 0.7924
IL-9	0.5501	0.588	715.8	52.38	52.63	0.3689 to 0.7313
IL-10	0.5526	0.5844	6.975	50	50	0.3652 to 0.7399
IL-12 (p70)	0.6445	0.1255	2.815	70.83	62.5	0.4524 to 0.8367
IL-12 (p40)	0.7346	0.0069	112.6	76.92	80	0.5803 to 0.8889
IL-16	0.6115	0.6115	183.0	61.9	63.16	0.4344 to 0.7887
IL-17	0.6377	0.1343	9.745	60.87	66.67	0.4647 to 0.8107
IL-18	0.7263	0.0155	116.5	65.22	70.59	0.5646 to 0.8881
TNF-alpha	0.6997	0.0334	61.74	66.67	66.67	0.5319 to 0.8676
TNF-beta	0.6302	0.1675	926.9	58.33	62.5	0.4561 to 0.8043

Abbreviations: IL, interleukin; TNF, tumor necrosis factor.

patients with severe COVID-19.[17,66–70] An analysis of 10 cohort studies involving a total of 1798 patients with severe COVID-19 revealed elevated IL-6 concentration in all patients.[35,71–76] The serum levels of IL-6 were determined at 517–796 pg/mL in patients with ARDS, and they decreased to 42.9–94.7pg/mL in convalescents. In another study, IL-6 levels were also markedly higher in 86.8% patients with severe COVID-19.[74]

The results of this study can be useful for differentiating patients in the early stage of SARS-CoV-2 infection and identifying patients whose health condition can deteriorate rapidly due to the excessive production of inflammatory cytokines. It should be noted that IL-6 has been included in the panel of laboratory tests for assessing the condition of COVID-19 patients, and similarly to TNFα, IL-6 concentration is determined routinely in many laboratories.[77,7879–82]The changes of serum concentrations of those proteins are really useful and help to diagnose and monitor the course of the disease patients with COVID-19.

In view of the above, new prognostic markers should be developed and included in routine clinical practice to effectively diagnose patients infected with the SARS-CoV-2 virus in different stages of the disease. The present findings indicate that the analyzed cytokines have potential diagnostic value for identifying patients with mild symptoms in the initial stage of COVID-19 and downregulating CS which has potentially life-threatening consequences. The research showed that IL-1ra, IL-2Rα, IL-6, IL-8, IL-12 p40, IL-16, and IL-18 levels serve as potential diagnostic biomarkers in COVID-19 patients. What is more, increased IL-1α levels proved to be valuable in assessing the severity of COVID-19.

Finally, some of limitations of our study are also worth considering. Our study has been conducted on a small group of patients (100 patients) divided into two smaller groups according to the MEWS scale. Moreover, our patients participating in the study were diagnosed with only stage 1 and stage 2 of COVID- 19. Further research should be carried out on a greater group of patients with COVID-19 including 4 stages of COVID-19. Our study

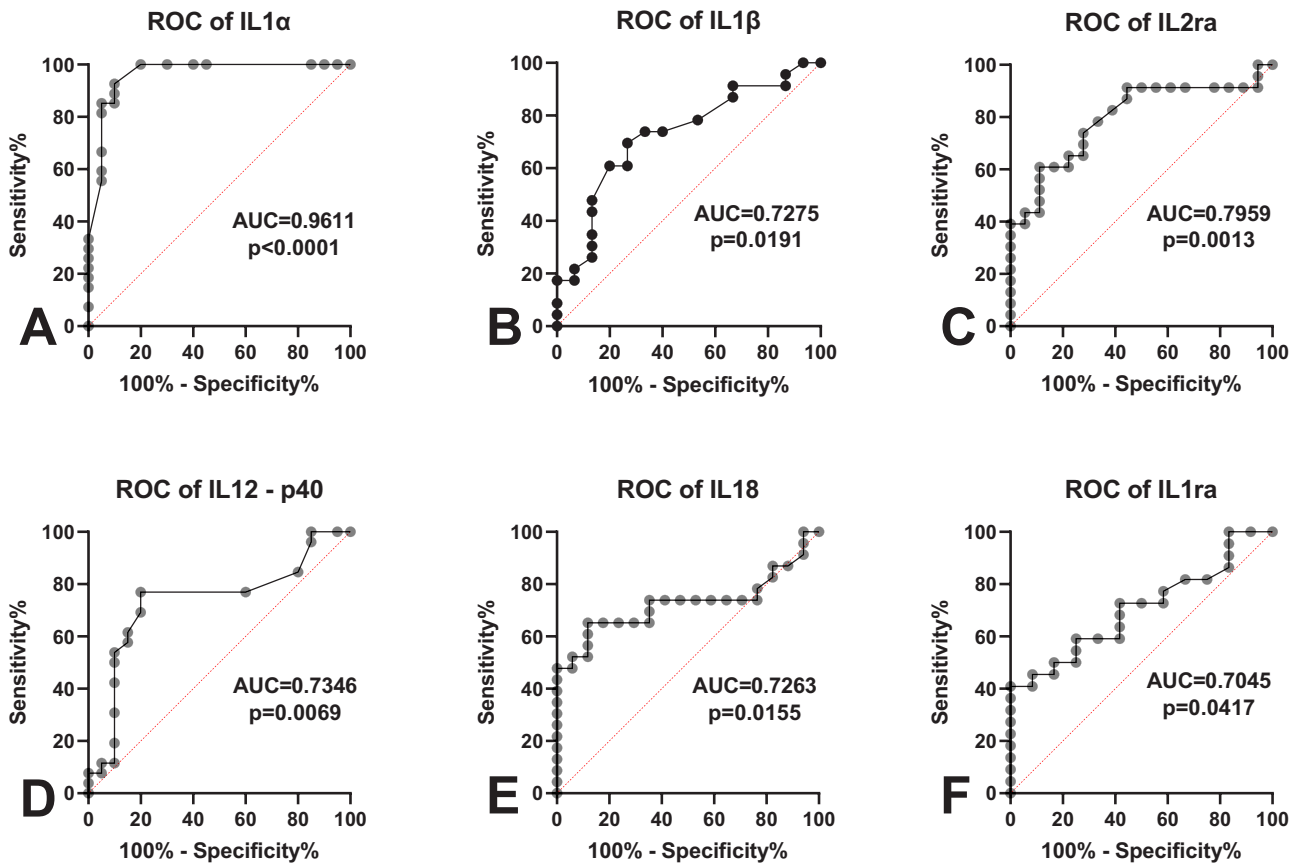

Figure 4 Receiver operating characteristic (ROC) curves of proinflammatory (**A**–**E**) and anti- inflammatory (**F**) cytokines for differentiating COVID-19 patients with different MEWS scores.

may be a pre-eliminary step for further clinical trials evaluating the chemokines and growth factors levels and their diagnostic utility in a larger population of patients with COVID-19.

Disclosure

The authors declare no conflicts of interest in this work.

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