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ALTERED EXPRESSION OF GENES RELATED TO INFLAMMATION AND COAGULATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF PATIENTS WITH SEVERE COVID-19

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus that causes coronavirus disease 2019 (COVID-19), the respiratory illness responsible for COVID-19 pandemic. Hyperinflammation and coagulopathy contribute to disease severity and death in patients infected with SARS-CoV-2. Gene expression analysis in peripheral blood mononuclear cells (PBMCs) is valuable to evaluate disease-associated and drug-response related genes. ***Aim.*** *In this study, we aimed to investigate the expression of genes related to inflammation and coagulation in PBMCs of patients with severe COVID-19.* ***Methods.*** *The gene expression in PBMCs (52 patients with severe COVID-19 and 59 healthy volunteers) was determined by RT-qPCR.* ***Results.*** *Overexpression of the OAS1, RNASEL, MX1, EIE2AK2, IL8, IL6, IL10, F5 genes and downexpression of the CD4 gene were found out in the PBMCs of SARS-CoV-2 infected patients compared to the healthy volunteers. Using the ROC curve, we found the genes with excellent (IL10, EIF2AK2, F5) and with good (FN1, MX1, RNASEL) diagnostic* AUC_{ROC}. ***Conclusions.*** *The results of this study designated the genes in PBMCs that can be potential candidate biomarkers for diagnosis of SARS-CoV-2-induced hyperinflammation, hypercoagulation, and the evaluation of therapy effectiveness for the patients with severe COVID-19.*

Keywords: *SARS-CoV-2, PBMCs, gene expression, hyperinflammation, hypercoagulation.*

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus that causes coronavirus disease 2019 (COVID-19), the respiratory illness responsible for an ongoing global COVID-19 pandemic [1]. The clinical course of COVID-19 varies from mild symptoms to acute respiratory distress syndrome, hyperinflammation, and coagulation disorder [2]. The patients with COVID-19 have a high risk of developing blood clots (thrombosis) mainly in their lungs, which leads to higher mortality rate [3]. Thus, of April 2024, the SARS-CoV-2-related mortality rate was 7,0 million people worldwide (https://www.worldometers.info/coronavirus/).

Currently, the main SARS-CoV-2 interventions are vaccines. Vaccines reduce the risk of COVID-19, including the risk of severe illness and death among people who are fully vaccinated. Vaccine effectiveness against hospitalizations has remained relatively high over time, although it tends to be slightly lower for older adults and for people with weakened immune systems. (https://www.cdc.gov/coronavirus/2019-ncov/vaccines/effectiveness/work.html). However, unvaccinated people, older adults, and people with weakened immune systems remain at risk of COVID-19 and will require effective treatment. An early diagnosis of SARS-CoV-2-induced dysregulation in the immune system would allow doctors to select effective therapy for patients and monitor the effectiveness of their treatment.

The hematopoietic system plays a key role in the observed hyperinflammation and coagulation disorder in COVID-19 ill patients [4]. Biochemical monitoring of inflammation and coagulation state in COVID-19 patients can be performed using following laboratory tests: C-reactive protein, erythrocyte sedimentation rate, lymphocyte count, D-dimer, prolonged prothrombin time [5—8]. However, diagnosing and assessing the effectiveness of treatment of the patients with severe COVID-19 using biochemical laboratory tests requires frequent and large blood sample collections, which can develop hospital-acquired anemia in patients [9—12]. Moreover, the D-dimer test does not always detect microthrombosis [13], which is a prominent clinical feature of COVID-19 and 91.3% of deceased patients had microthrombosis in the capillaries of the lungs, heart, or other organs [15—16]. The volume of microthrombi is small compared to massive venous thromboses, so the amount of fibrin degradation products entering the systemic circulation may not be sufficient to increase D-dimer [13] significantly. There were also identified the cases of COVID-19-induced thrombosis with a slight increase or within the normal range of D-dimer [17, 18]. Therefore, the search for new candidate biomarkers for diagnosing SARS-CoV-2-induced immune dysregulation (hyperinflammation and hypercoagulation) and evaluating therapy for the patients with severe COVID-19 is relevant.

Gene expression analysis in peripheral blood mononuclear cells (PBMCs) is valuable to evaluate the disease-associated [19—22] and drug-response related genes [23]. Additionally, gene expression analysis enables the determination of multiple genes in a single blood sample. In this study, we investigated the expression of genes related to inflammation and coagulation in PBMCs of the patients with severe COVID-19. In current research, we found overexpression of the *OAS1, RNASEL, MX1, EIE2AK2, IL8, IL6, IL10, F5* genes and downexpression of the *CD4* gene in PBMCs of the patients with severe COVID-19. ROC analysis of the expression data identified the *EIF2AK2, IL10, RNASEL, MX1 F5, FN1* genes in PBMCs that classify SARS-CoV-2-infected and healthy patients with good accuracy and that can be potential biomarkers of hyperinflammation and hypercoagulation in the patients with severe COVID-19.

Materials and Methods

Sample collection, processing and storage

Whole blood samples of the patients with severe COVID-19 (COVID-19 group, n = 52) were collected by the Center for Public Health of the Min-

istry of Health of Ukraine (Kyiv, Ukrainian). All patients had acute respiratory symptoms and were positive for SARS-CoV-2 by a specific qPCR test. Control group was whole blood samples of the healthy volunteers (n = 59) that were collected by Feofaniya Clinical Hospital (Kyiv, Ukraine). Sampling was conducted in accordance with the Helsinki declaration and approved by the Ministry of Health of Ukraine in agreement of the Center for Public Health with Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine.

Whole blood samples were collected into EDTA tubes (Vacusera, Turkey) to prevent blood clotting. PBMCs were extracted from the buffy coat by centrifugation of the whole blood sample at 2000 *rpm* for 15 min at +4 °C. TRI-Reagent (Zymo Research, USA) was added to extracted PBMCs (1 : 3) to prevent RNA degradation and mixed well using Vortex. The PBMCs samples were stored immediately at –80 °C.

Real-Time qPCR Assay

Total RNAs were extracted from PBMCs samples by using a Direct-zol RNA Miniprep Plus (Zymo Research, USA), according to the protocol suggested by the manufacturer. RNA integrity was analyzed in the Microchip electrophoresis system (MCE-202/MultiNA SHIMADZU, Germany) by using a RNA reagent kit for MultiNA (SHIMADZU, Germany), SYBR Green II RNA gel stain (Life Technologies, USA) та RNA 6000 Ladder (Life Technologies, USA)). Total RNAs were quantified spectrometrically, and RNA purity was assessed by the 260/280 nm ratio on a MaestroNano Pro Micro-Volume MN-913 spectrophotometer (MAESTROGEN, Taiwan).

cDNA was synthesized from every total RNA sample by using a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA). Reverse transcription was conducted using 1 μg total RNA per sample and the protocol included incubating for 10 min at 25 °C followed by 120 min at 50 °C. Terminate the reaction by heating at 85 °C for 5 min.

mRNA levels of the studied genes were quantified by a Thermal Cycler CFX96 Real-Time system (BIO-RAD, Singapore) using a HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia). Human cDNA was amplified with the gene-specific primers (Table 1). The primers sequenced were designed on GenBank database and were synthesized (Invitrogen, USA). Average fold change values were determined by the $2^{(\Delta Ct)}$ method [24]. The mRNA level of all investigated genes were normalized to *TBP* mRNA as a control and expression of *TBP* mRNA was taken as 100 expression units. The data were presented as mean ±SD.

Statistical analysis

The Mann-Whitney U test and ROC curve were performed in Prism version 8.0.1. (GraphPad, La Jolla, CA) for independent data samples. The normality of the distribution was checked according to the Shapiro-Wilk test [25].

Results and Discussion

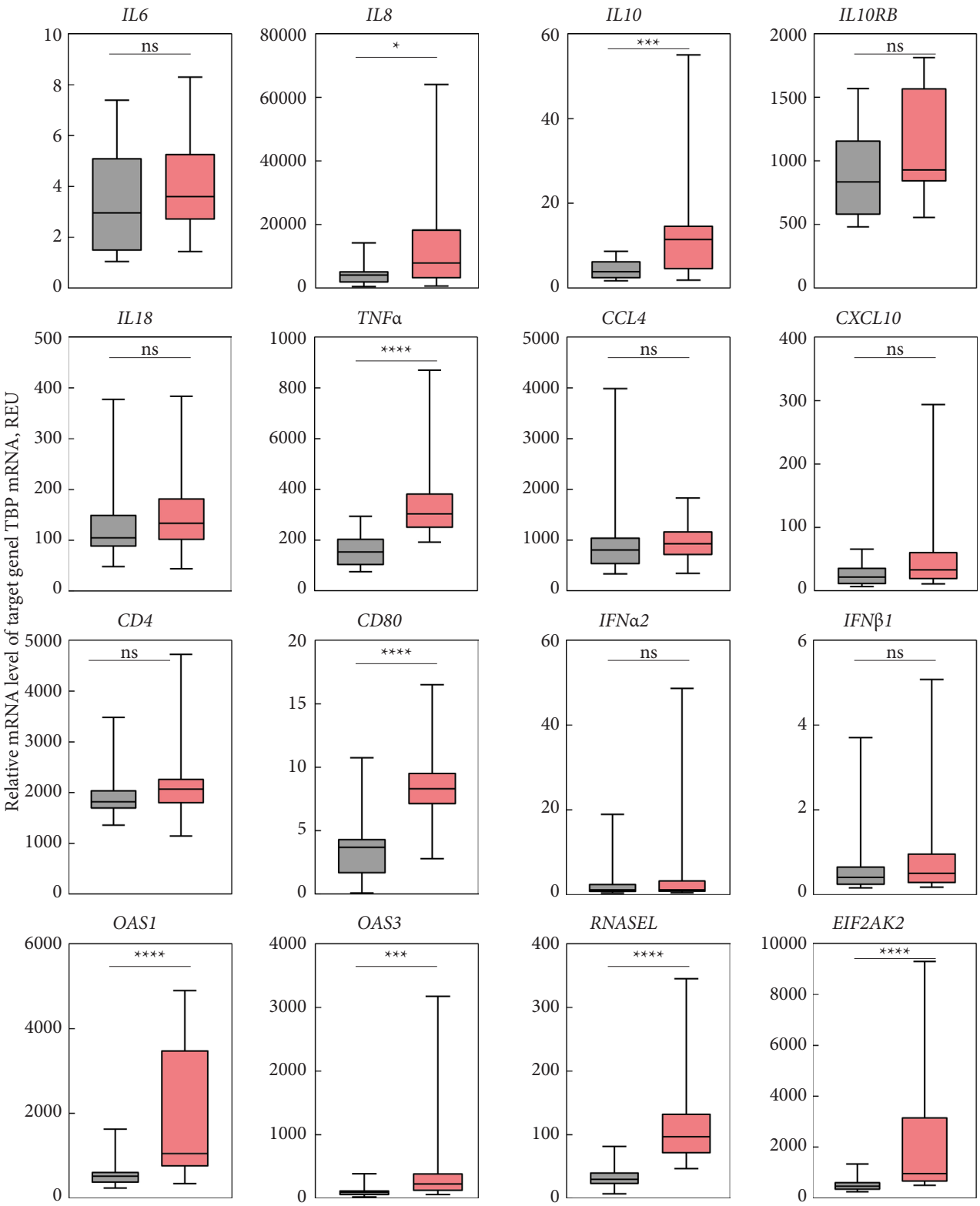
Altered expression of the investigated genes in PBMCs of patients with severe COVID-19

Dysregulation of the immune system can lead to an inappropriate local and systemic immune responses and subsequently the rapid spread of the virus, leading to severe COVID-19 disease. Therefore, recognizing and improving the immune system disorder should always be part of the diagnosis and treatment protocols of patients with COVID-19 [26]. Therefore, analysis of the gene expression profiles of peripheral blood cells can help us to understand a patient's condition [27—28] and can be potentially a novel tool for the diagnosis of immune system disorder in the patients with severe COVID-19.

During this research, the goal was to select a small number of genes that could be used for a diagnostic panel describing the patient's condition. At first in our study, we analyzed the mRNA expression of the *IL6, IL8, IL10, IL10RB, IL18, TNF*α,

Table 1. **Sequences of primers used for RT-qPCR**

Gene	Primer	Sequence (5' → 3')
IL6	Forward	GATTCAATGAGGAGACTTGCC
(interleukin 6)	Reverse	TGTTCTGGAGGTACTCTAGGT
IL8	Forward	ACTCCAAACCTTTCCACCC
(interleukin 8)	Reverse	CAATAATTTCTGTGTTGGCGC
IL10	Forward	AAGACCCAGACATCAAGGC
(interleukin 10)	Reverse	AAGAAATCGATGACAGCGC
IL10RB	Forward	AACAACCCATGACGAAACG
(interleukin 10 receptor, beta)	Reverse	TCTTGTAAACGCACCACAG
IL18	Forward	TGACCAAGTTCTCTTCATTGAC
(interleukin 18)	Reverse	GGTGCATTATCTCTACAGTCAG
*TNF*α	Forward	CTCTAATCAGCCCTCTGGC
(tumor necrosis factor alpha)	Reverse	GAGGGTTTGCTACAACATGG
CCL4	Forward	AGCTGTGGTATTCCAAACC
(chemokine (C-C motif) ligand 4)	Reverse	TCATACACGTACTCCTGGAC
CXCL10	Forward	ACGTGTTGAGATCATTGCT
(chemokine (C-X-C motif) ligand 10)	Reverse	AAATTCTTGATGGCCTTCGA
CD4	Forward	CCTCCTGCTTTTCATTGGGCTAG
(T-cell surface glycoprotein CD4)	Reverse	TGAGGACACTGGCAGGTCTTCT
CD80	Forward	CACTTCTGTTCAGGTGTTATCC
(CD80 molecule)	Reverse	AACAGAAACATTGTGACCACAG
*IFN*α*2*	Forward	TCCATGAGATGATCCAGCAG
(interferon alpha 2)	Reverse	CAAGCAGCAGATGAGTCCT
*IFN*β*1*	Forward	GATTCCTACAAAGAAGCAGCA
(interferon beta 1)	Reverse	CTCCCATTCAATTGCCACAG
OAS1	Forward	TCCAAGGTGGTAAAGGGTG
(2'-5' oligoadenylate synthetase 1)	Reverse	TGAGGAAGACAACCAGGTC
OAS3	Forward	AAACTGTCAAGGGAGGCTC
(2'-5' oligoadenylate synthetase 3)	Reverse	AGCAGTCGAGGAAGATGAC
RNASEL (2'-5' -oligoisoadenylate synthetase-dependent ribonuclease L)	Forward	ATCTAGAGGACCTTGGACG
	Reverse	TACTTTGAGCTTTCAGATCCTC
EIF2AK2 (eukaryotic translation initiation factor 2-alpha kinase 2)	Forward	ACATACCGTCAGAAGCAGG
	Reverse	GAAATGTAAACCTCCTATCATGTGG
MX1	Forward	TAATAAAGCCCAGAATGCCA
(MX dynamin-like GTPase 1)	Reverse	TTAGAGTCAGATCCGGGAC
ARG2	Forward	ACAATACAGGGTTGCTATCAG
(arginase type II)	Reverse	TAGTCTTCGCCTCTTCCTC
NOS2	Forward	ATGACCTTCAGTATCACAACCT
(nitric oxide synthase 2, inducible)	Reverse	CTGGAGACTTCTTTCCCGT
XDH	Forward	GGACAGTTGTGGCTCTTGAGGT
(xanthine dehydrogenase)	Reverse	GGAAGGTTGGTTTTGCACAGCC
F5	Forward	FGCCAGACCTTGCTGGAAAATGG
(coagulation factor V)	Reverse	CCAACCTCTGTGTTTAGGAGCC
F10	Forward	TGG TGG AAC CAT TCT GAG CGAG
(coagulation factor X)	Reverse	CGG TTG TGC TTG ATG ACC ACCT
FN1	Forward	ACAACACCGAGGTGACTGAGAC
(fibronectin 1)	Reverse	GGACACAACGATGCTTCCTGAG
TBP	Forward	TGTATCCACAGTGAATCTTGGTTG
(TATA-box binding protein)	Reverse	GGTTCGTGGCTCTCTTATCCTC


IL6
IL8
IL10
IL10RB
IL18
TNFα
CCL4
CXCL10
CD4
CD80
IFNα2
IFNβ1
OAS1
OAS3
RNASEL
EIF2AK2
ns
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Relative mRNA level of target genel TBP mRNA, REU

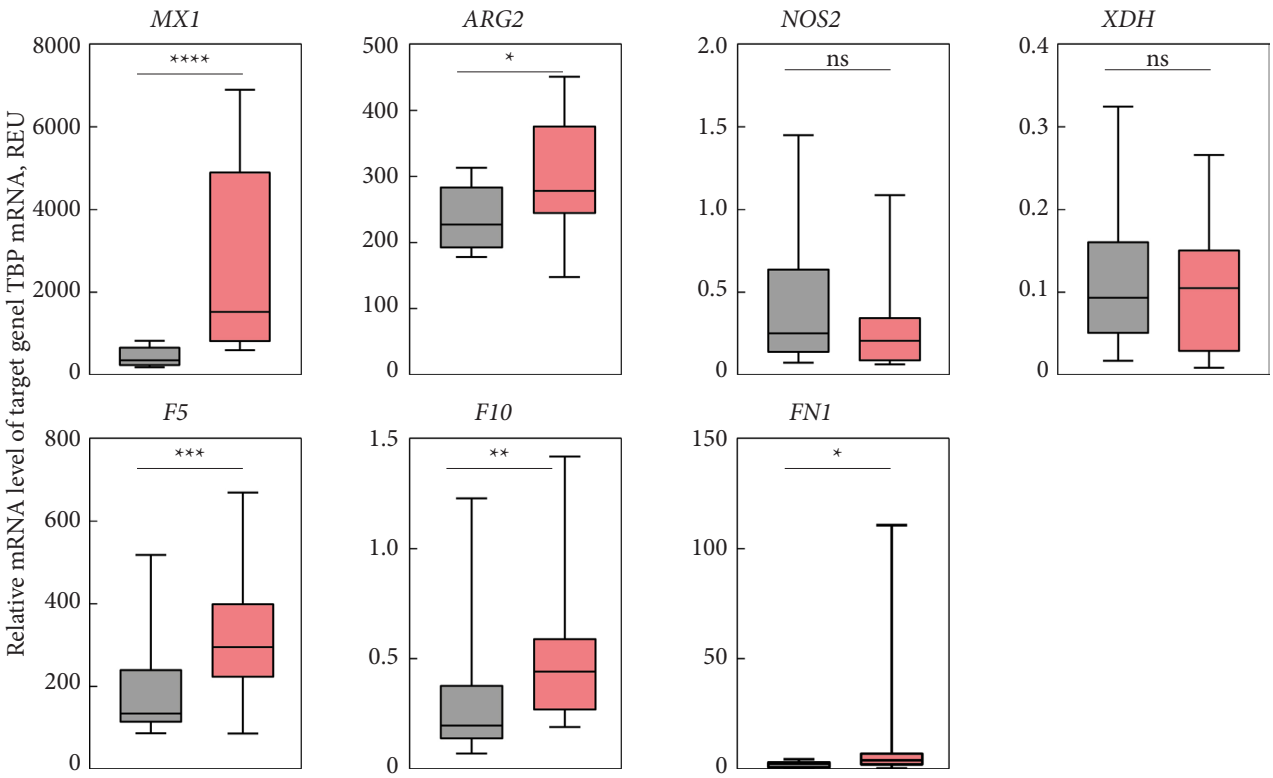

Fig. 1. The mRNA level of studied genes in PBMCs of patients with severe COVID-19: Control (left box on each box chart) — a group of healthy volunteers, $n = 20$; COVID-19 (right box on each box chart) — a group of patients with severe COVID-19, $n = 20$: * — $P < 0.05$ vs Control; ** — $P < 0.01$ vs Control; *** — $P < 0.001$ vs. Control; **** — $P < 0.0001$ vs. Control; ns — statistically not significant vs. Control; REU — relative expression units

CCL4, CXCL10, CD4, CD80, OAS1, OAS3, RNASEL, MX1, EIF2AK2, IFNα2, IFNβ1, ARG2, NOS2, XDH, F5, F10, FN1 genes in a small study groups (Control group $n = 20$ and COVID-19 group $n = 20$) (Fig. 1). Increased mRNA level of the *IL8* ($P < 0.05$), *IL10* ($P < 0.001$), *TNFα* ($P < 0.0001$), *CD80* ($P < 0.0001$), *OAS1* ($P < 0.0001$), *OAS3* ($P < 0.01$), *RNASEL* ($P < 0.0001$), *MX1* ($P < 0.0001$), *EIF2AK2* ($P < 0.0001$), *ARG2* ($P < 0.05$), *F5* ($P < 0.001$), *F10* ($P < 0.01$), *FN1* ($P < 0.05$) genes was found in PBMCs of the patients with severe COVID-19 group compared to the control group. Therefore, based on the research results, we selected the *IL8, IL10, TNFα, OAS1, RNASEL, MX1, EIF2AK2, F5, FN1* genes to continue investigation of the mRNA expression of genes in a larger study groups (control group $n = 59$ and COVID-19 group $n = 52$). Despite the fact that the mRNA level of the *IL6, CD4* genes remained unchanged in PBMCs of the patients of COVID-19 group ($n = 20$) compared to the control group ($n = 20$) we decided to continue studding expression of this genes because they are promising biomarker considering the literature [29].

Hyperinflammatory state of the patients with severe COVID-19, which contributes to the occurrence of fatal complications and poor prognosis, is associated with the uncontrolled release of cytokines [30—31]. Up-regulated mRNA level of

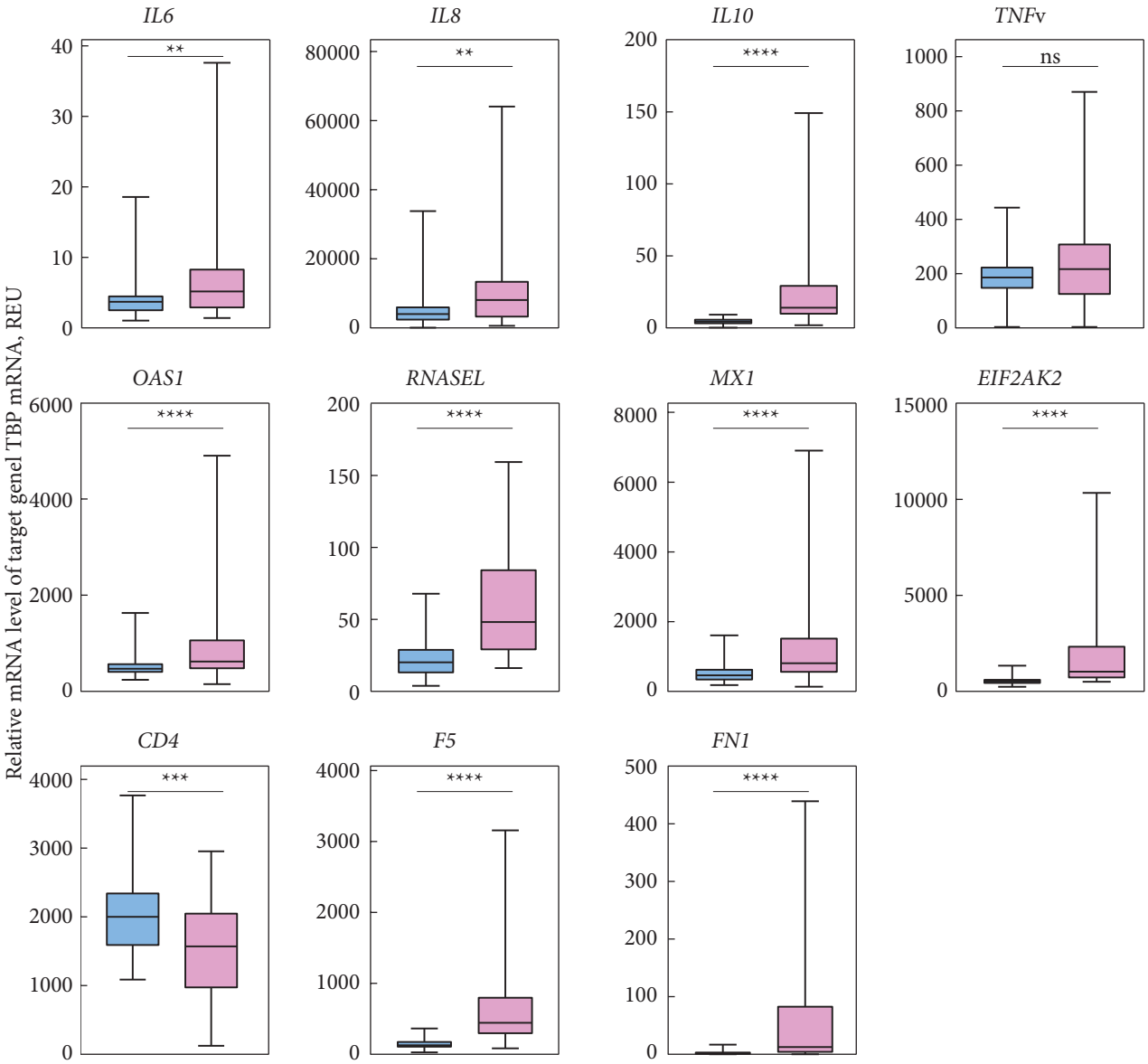

Fig. 2. The mRNA level of the *IL6, TNFα, IL8, IL10, OAS1, RNASEL, MX1, EIF2AK2, CD4, F5* and *FN1* genes in PBMCs of the patients with severe COVID-19: Control (left box on each box chart) — a group of healthy volunteers, $n = 59$; COVID-19 (right box on each box chart) — a group of patients with severe COVID-19, $n = 52$: ** — $P < 0.01$ vs Control; *** — $P < 0.001$ vs. Control; **** — $P < 0.0001$ vs. Control; ns — statistically not significant vs. Control; REU — relative expression units

cytokines *IL8* ($P < 0.01$), *IL6* ($P < 0.01$), *IL10* ($P < 0.0001$) was found in PBMCs of the patients of COVID-19 group ($n = 52$) compared to the control group ($n = 59$) (Fig. 2). However, the relative expression of the TNFα gene remained statistically unchanged in the patients with severe COVID-19 compared to the control group. Cytokine overproduction causes the antiviral state of the cell by

stimulating the expression of thousand genes, which are known as interferon-stimulated genes (OAS/RNase L system, MX1 protein and PKR) [32—33]. The mRNA levels of *OAS1* ($P < 0.0001$), *RNASEL* ($P < 0.0001$), *MX1* ($P < 0.0001$), *EIF2AK2* ($P < 0.0001$) in PBMCs of the patients with severe COVID-19 ($n = 52$) were increase in compared to the control group ($n = 59$) (Fig. 2). Overexpression of the *OAS1*, *RNASEL*, *MX1*, *EIF2AK2, IL8*, *IL6*, *IL10* genes induced by SARS-CoV-2 in PBMCs indicates the hyperinflammation state in the patients with severe COVID-19 [31].

Virus-induced dendritic cells migrate to regional lymph nodes, activating cytotoxic (CD8+), helper (CD4+) T, and rare memory T cells, which are able to induce an adaptive immune response at respiratory infection [34]. Both lymphocytosis and lymphopenia were found to be associated with SARS-CoV-2 [35—37]. While lymphocytosis indicates an active anti-viral response, lymphopenia is a sign of poor prognosis [38]. CD4 is a glycoprotein that high expressed on CD4 T helper cells and expressed at lower levels on monocytes and some neutrophils [39—41]. We found out downregulation of the *CD4* mRNA level ($P < 0.001$) in the PBMCs of patients with severe COVID-19 ($n = 52$) compared to the control group ($n = 59$) (Fig. 2). Decreased *CD4* expression in the PBMCs can be associated with lymphopenia and the *CD4* gene can be potential biomarker to indicate lymphopenia in the patients with COVID-19.

The patients with COVID-19 have a higher risk of developing blood clots, mainly in the lungs, which is associated with higher mortality [42]. The ~70% of COVID-19 patients, who died, had disseminated intravascular coagulation [43]. The blood clots can be in both venous and arterial circulations, and in small blood vessels. Hypercoagulable state in the patients with COVID-19 has been confirmed in a number of studies that have shown higher levels of D-dimer, fibrinogen, and fibrinogen breakdown products [5], prolonged prothrombin time, international normalized ratio, and thrombin time in the SARS-CoV-2 infected patients [31]. Overexpression of the *F5* and *FN1* genes ($P < 0.0001$) was found in the PBMCs of patients with severe COVID-19 ($n = 52$) compared to a group of healthy volunteers ($n = 59$) (Fig. 2). Upregulation the *F5* and *FN1* genes may indicate an increased coagulation state in the patients with severe COVID-19 [5—6].

Receiver operating characteristics (ROC) curves and potential biomarkers

The ability of biomarkers to predict a disease is commonly evaluated using ROC curves. Therefore, we assessed the accuracy and reliability of the potential gene biomarkers to detect SARS-CoV-2-induced hyperinflammation, hypercoagulation, and lymphopenia, plotting ROC curves and calculated AUCs with a 95% confidence interval. The diagnostic parameter of ROC analysis is the area under the ROC curve (AUC_{ROC}) [44]. Among the investigated genes, ROC-analysis of relative gene expression in PBMCs of patients with severe COVID-19 revealed an excellent diagnostic AUC_{ROC} for the IL10 ($AUC_{ROC} = 0.91$), EIE2AK2 ($AUC_{ROC} = 0.9$), and F5 ($AUC_{ROC} = 0.95$) genes (Fig. 3). Besides, *FN1* ($AUC_{ROC} = 0.86$), *MX1* ($AUC_{ROC} = 0.78$), *RNASEL* ($AUC_{ROC} = 0.85$) could also be predictive biomarkers. The AUCs of IL6 ($AUC_{ROC} = 0.67$), IL8 ($AUC_{ROC} = 0.67$), OAS1 ($AUC_{ROC} = 0.71$), CD4 ($AUC_{ROC} = 0.68$) genes were less than 0.75 indicating their low diagnostic value. The obtained results of the pre-screening suggest that the *IL10, EIF2AK2, MX1, RNASEL* genes can be promising candidate biomarkers to indicate hyper-cytokinemia and the *F5, FN1* genes — hypercoagulable state in the patients with severe COVID-19.

Conclusions

This study identified nine genes (*IL6, IL8, IL10, OAS1, RNASEL, MX1, EIF2AK2, F5, FN1*) that are expressed at higher levels and the downexpressed CD4 gene in the PBMCs of the patients with severe COVID-19 compared to the healthy volunteers. We demonstrated the genes with the excellent (*IL10, EIF2AK2, F5)* and good (*FN1, MX1, RNASEL)*

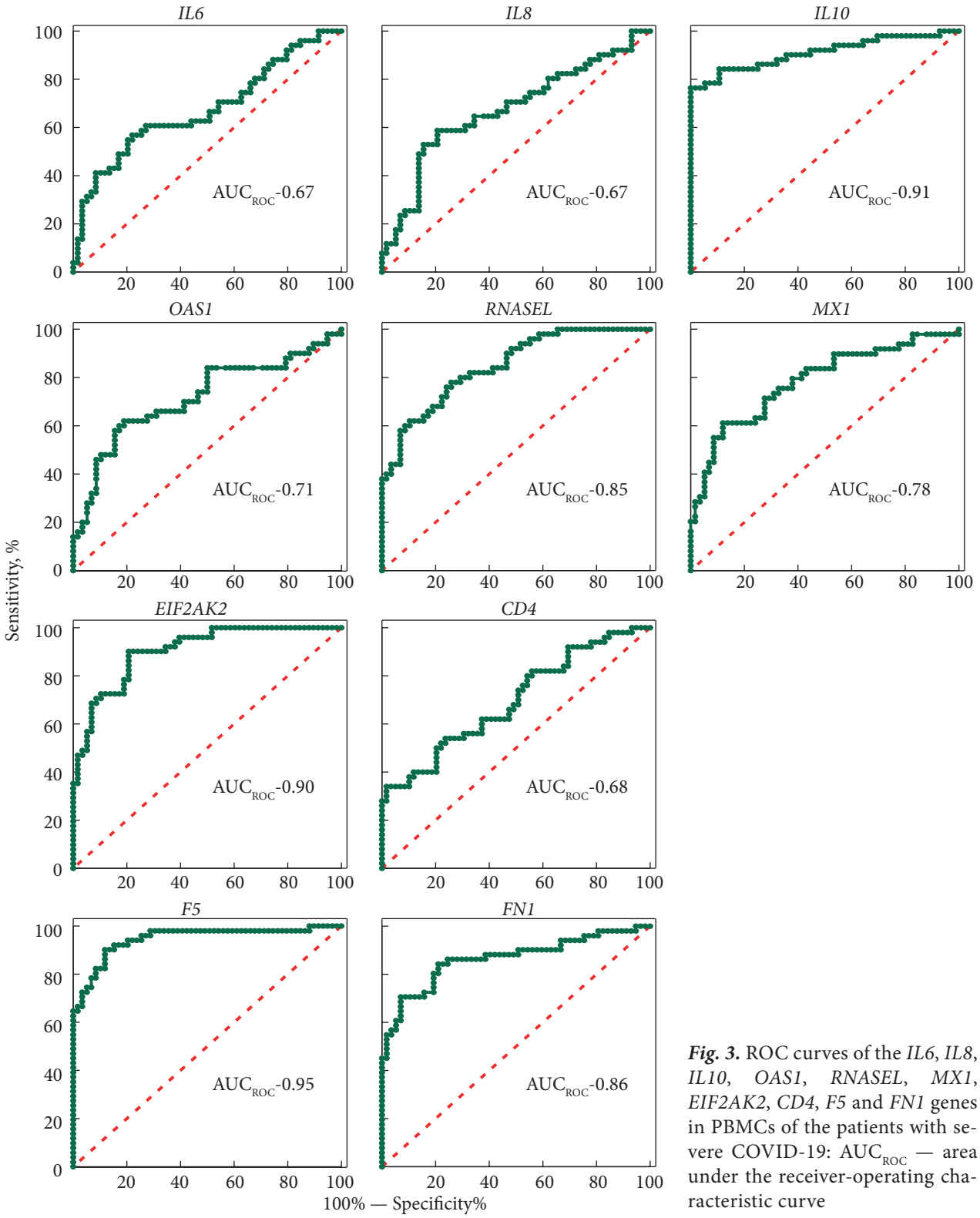

Fig. 3. ROC curves of the *IL6*, *IL8*, *IL10*, *OAS1*, *RNASEL*, *MX1*, *EIF2AK2*, *CD4*, *F5* and *FN1* genes in PBMCs of the patients with severe COVID-19: AUC$_{ROC}$ — area under the receiver-operating characteristic curve

diagnostic AUC_{ROC}. Overall, our results of prescreening are an important first stage towards identifying candidate biomarkers in the PBMCs for the diagnosis of hyperinflammation and hypercoagulation of the patients with severe COVID-19.

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ЗМІНЕНА ЕКСПРЕСІЯ ГЕНІВ ПОВ'ЯЗАНИХ ІЗ ЗАПАЛЕННЯМ ТА КОАГУЛЯЦІЄЮ В МОНОНУКЛЕАРНИХ КЛІТИНАХ ПЕРИФЕРІЙНОЇ КРОВІ ПАЦІЄНТІВ З ВАЖКИМ ПЕРЕБІГОМ COVID-19

Тяжкий гострий респіраторний синдром коронавірусу 2 (SARS-CoV-2), вірус, що спричиняє коронавірусну хворобу 2019 (COVID-19), респіраторне захворювання, що відповідає за пандемію COVID-19. Гіперзапалення та коагулопатії сприяють тяжкості захворювання та смерті пацієнтів, інфікованих SARS-CoV-2. Аналіз експресії генів у мононуклеарних клітинах периферичної крові (МКПК) є цінним для оцінки генів, пов›язаних із захворюванням та реакцією на ліки. ***Мета.*** У цьому дослідженні ми мали на меті дослідити експресію генів, пов›язаних із запаленням та коагуляцією у МКПК пацієнтів з гострою формою COVID-19. ***Методи.*** Експресію генів у МКПК (52 пацієнтів з тяжкою формою COVID-19 та 59 здорових добровольців) визначали за допомогою RT-qPCR. ***Результати.*** Виявлено надмірну експресію *OAS1, RNASEL, MX1, EIE2AK2, IL8, IL6, IL10, F5* генів та знижену експресію гена *CD4* у МКПК пацієнтів, інфікованих SARS-CoV-2, порівняно зі здоровими добровольцями. Використовуючи ROC-криву, ми виявили гени з відмінним (*IL10, EIF2AK2, F5*) та з добрим (*FN1, MX1, RNASEL*) діагностичним AUC_{ROC}. ***Висновки.*** Результати цього дослідження вказують на гени в МКПК, які можуть бути потенційними кандидатами на біомаркери для діагностики гіперзапалення, гіперкоагуляції, викликаних SARS-CoV-2, та оцінки ефективності терапії у пацієнтів з тяжким перебігом COVID-19.

Ключові слова: SARS-CoV-2, МКПК, експресії генів, гіперзапалення, коагулопатії.