SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the prognostic role of new laboratory biomarkers in severe COVID-19 disease

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1. Introduction and literature review

COVID-19 is a primary respiratory but potentially systemic inflammatory infectious disease caused by SARS-CoV-2 beta coronavirus. Since the outbreak of the COVID-19 disease, according to the recent report of World Health Organization, 664.8 million confirmed infected cases and 6.7 million confirmed deaths were identified all around the word. Most infected patients have mild or no symptoms, however, 13.8% of patients have severe disease, while 4.7% of these subjects may suffer from critical illness with respiratory and multiorgan failure. In certain cases, despite adequate hospital treatment the above symptoms may progress into organ dysfunction affecting the liver, kidneys, central nervous system, gastrointestinal tract and hematopoietic tissue. In addition, thromboembolic complications can manifest, which ultimately lead to the early death of such COVID-19 patients. The causative pathogen is a relatively large, enveloped, single-stranded RNA-carrying virus, which probably first became available via zoonotic disease, and then spread from person to person through numerous mutations.
The well-known risk factors for infection with the SARS-CoV-2 virus and the development of more serious clinical conditions are the advanced age and the male gender, as well as certain co-morbidities, especially disorders associated with endothelial cell damage, such as high blood pressure, other cardiovascular diseases, diabetes mellitus or chronic kidney failure, which not only increase the risk of infection, but are also independent predictors of a more severe disease prognosis.
For the laboratory detection of SARS-CoV-2, we primarily use sample from the upper respiratory tract. In infected patients, viral RNA can be detected quite early, even at the onset of symptoms by RT-PCR test. The antigen-based rapid test is based on the detection of the viral protein present in the sample. SARS-CoV-2 infection can be also confirmed even after one month following the presumed infection by measuring the humoral immune response to infection, when IgG, IgM or IgA antibodies against the SARS-CoV-2 are detected.

The clinical manifestations of COVID-19

Spreading with a droplet infection, the main entrance gate of the SARS-CoV-2 are the epithelial cells of respiratory tract and type II pneumocytes. After the internalization of the virus and its replication within cells, severe damage to the endothelium and vasculature can occur, which leads to increased vascular permeability, thus the

formation of alveolar and interstitial edema. In the early so-called exudative phase, the amount of surfactant produced by type II pneumocytes decreases, and fibrin deposits with hyaline membrane formation are observed. As a result of extensive tissue inflammation and massive cell damage, apoptosis at cellular level and necrotic processes at tissue level are initiated, which result in diffuse alveolar damage and significant deterioration of lung function. At the same time, the pathological proliferation of fibroblasts begins, which together with alveolar exudative processes and the direct damage of vascular structures caused by the virus lead to irreversible pulmonary damage mediated by many cytokines (e.g. TGF-β) and interleukins (e.g. IL-1β), in total, can lead to acute respiratory distress syndrome (ARDS). Meanwhile, because of the activation of endothelial cells, the expression of pro- and anti-inflammatory mediators, as well as neutrophils and monocytes are significantly increased in the capillaries of the lungs. Increased ratio of atelectasis and consolidated lung areas with the disturbance of the blood supply to the lungs and the thrombotic and immune-mediated obstruction of the capillaries, along with the ventilation-perfusion disproportion due to the formation of shunt circulation can result in a dramatic deterioration of the gas exchange.

Other secondary organ involvement is common in patients with COVID-19-associated ARDS. Among the disorders affecting the cardiovascular system, myo/pericarditis, cardiac ischemia and arrhythmia are the most likely alterations, moreover, in 20% of cases life-threatening malignant arrhythmias or cardiac arrest may occur. In patients infected with SARS-CoV-2 in a critical clinical condition, kidneys can often be affected, and even acute renal damage requiring replacement therapy can be developed. The high rate of kidney involvement can be explained by several mechanisms of injury, including reduced blood flow, the presence of glomerular microthrombi, and the activation of the renin-angiotensin-aldosterone (RAAS) autocrine system. One-third of critically ill patients may have neurological manifestations, such as inflammatory symptoms in the central and peripheral nervous system (e.g. myelitis, meningoencephalitis, encephalopathy, etc.).

The molecular pathomechanism of COVID-19 disease

The major receptor of SARS-CoV-2 is the angiotensin-converting enzyme-2 (ACE2), a transmembrane glycoprotein with metalloprotease activity, located on the cell surface. In humans, ACE2 is mainly expressed on the surface of nasal and bronchial epithelium

and in the membrane of type II pneumocytes. Recent studies showed its high expression on the surface of other human tissues as well. The position of the receptor apparently determines the organotropism of the coronavirus and can explain the symptoms of the infection. The SARS-CoV-2 virus contains four proteins encoded by the genome. The M ("membrane") protein is the most important structural component of the outer envelope, the E ("envelope") protein is a much smaller part present in the envelope, and it plays a role in the assembly of virus particles and their exit from the host cell, the N ("nucleocapsid") protein is the component of the inner shell surrounding the genetic material. In terms of infectivity, the S ("spike") protein protruding from the outer shell has the greatest importance. Coronavirus owns a structural protein S (spike) on its surface that is composed of two subunits named S1 and S2, and it is responsible for viral binding to host cells. The binding to the ACE2 causes a conformational change in protein S1/S2 and provokes endocytosis. Parallelly, an amino acid sequence is released from the protein S, stimulating certain intracellular proteases to allow the fusion between the cell membrane and the viral envelope. TMPRSS2 (transmembrane protease serine 2) seems to have an outstanding role in this proteolytic process: recent clinical studies demonstrated that the inhibition of the molecule blocked the viral endocytosis and consequently the infection of the cell. Nevertheless, recent *in vitro* investigation found a very low human endothelial ACE2 expression and an ineffective upregulation of the protein in cytokine environment compared to the expression on the epithelium of the airways. These findings discredit the hypothesis of direct viral infection and intracellular viral replication in the endothelium. At this moment, the indirect endothelial activation via the ancient pathogen-associated patterns seems to be more likely.

The role of ACE2 in the progression of COVID-19 disease

ACE2 is an enzyme with metalloprotease activity, acting as one of the key molecules of the RAAS responsible for homeostasis. As a result of renal hypoperfusion, hyponatremia or activation of the sympathetic nervous system, pro-renin is formed in the glomerular cells of the kidney, which is converted into active renin either locally or by binding to the pro-renin receptor expressed by other tissues. Angiotensinogen produced by the liver is transformed by renin into angiotensin I (Ang I). Ang I is a substrate of the ACE produced in the lungs, from which angiotensin II (Ang II) is formed, which as a potent end product regulates a number of processes through

angiotensin receptors 1 and 2 (AT1R and AT2R), which are diffusely present in the human body: it causes arterial and venous vasoconstriction, hypertension and tachycardia in the vascular system, aldosterone release in the adrenal cortex, consequent sodium retention and potassium excretion in the distal tubules of the kidney, while by stimulating the hypothalamus and pituitary gland, it leads to an increase in antidiuretic hormone (ADH) levels and a feeling of thirst.

In the last few decades, it has become clear that Ang II also promotes the activation of proinflammatory genes via the AT1R, MAPK and Rho proteins in the signal transduction pathway mediated by NF-κB pathway. These promote an increase in vascular permeability, and cause the production of many cytokines and chemokines, as well as cell adhesion molecules, e.g. Vascular cell adhesion protein-1 (VCAM-1), which together induce the migration of cell types involved in inflammatory processes. In addition, Ang II increases the expression of platelet adhesion molecules (e.g. P-selectin) and tissue factor (TF) and plasminogen activator inhibitor-1 and 2 (PAI-1, PAI-2) by endothelial cells and smooth muscle cells in the local, which can also increase the probability of thrombosis. The protective effects through AT2R counterbalance the proinflammatory and coagulation processes induced via the activation by AT1R. Activation of the receptor increases the production of nitric oxide (NO) in endothelial cells and inhibits the activation of the NF-κB mediated signaling cascade within certain cells.

The substrate of the ACE2 enzyme is Ang I and Ang II, from which angiotensin 1-9 (Ang 1-9) and angiotensin 1-7 (Ang 1-7) are formed through proteolytic cleavage. The latter binds to the mitochondrial assembly receptor (MASR), which causes vasodilation, thereby reducing blood pressure, and inhibits the thrombotic, phlogistic and profibrotic processes of the vessel wall, thus functioning as a kind of internal counterbalance against extensive Ang II formation.

In certain disease states with chronic inflammation, ACE2 is cleaved by a protein with protease activity (A disintegrin and metalloproteinase 17, ADAM17) and hence it circulates in the bloodstream. As a result of the cleavage, the function of ACE2 is damaged and it loses its balancing ability. This is supported by those observations that the amount of circulating ACE2 significantly increases in the blood samples of patients with different cardiovascular diseases, such as hypertension, aortic stenosis, heart failure and atrial fibrillation.

In case of SARS-CoV-2 infection, both "arms" of the RAAS (Ang II/AT1R/NF-κB pathway induction) and Ang 1-7/Ang 1-9/AT2R/NF-κB pathway inhibition) work extensively. ACE2, which is still on the cell surface, is cleaved from the cell surface by the enzyme ADAM17 and solubilized. The amount of Ang II increases due to the decreased activity of its breaking down enzyme (ACE2). Overall, the reduction of ACE2 expression can lead to a decrease in the protective cardiovascular effect, and ultimately to a first local and then generalized increase in the ratio of proinflammatory and procoagulant effects. At the same time, the binding of the released ACE2 protein and the virus particles to each other via the S-protein can presumably have a positive effect, as it can inhibit virus replication.

Based on the mechanisms detailed above, we would expect an increase in the amount of soluble ACE2 in severe COVID-19 disease, but contradictory results were reported in former studies published on this field. Prior to the present investigation, our group had detected elevated serum activity of soluble ACE2 during the follow-up of a critically ill COVID-19 patient. Therefore, we considered the great importance of further examination these preliminary results on a large group of patients.

The potential role of HE4 in COVID-19 disease

HE4 is a member of the whey acidic protein (WAP) family, the product of the *WFDC2* gene. The WAP protein family includes proteins with antiprotease and antibacterial activity and anti-inflammatory effects, which are produced in large quantities in the lungs and play a significant role in preventing the proteolytic processes of pathogens. Secretory leucocyte protease inhibitor (SLPI) and elafin are two prominent members of the group, which primarily through their inhibitory effect on neutrophil elastase, inhibit the growth of certain bacteria (e.g. *Pseudomonas aeruginosa*, *Staphylococcus aureus*) and fungi (e.g. *Aspergillus fumigatus, Candida albicans*), and can also provide an effective protection against retroviruses (e.g. *Human Immunodeficiency Virus - HIV*).

HE4 is a small secretory glycoprotein that first became known due to its supposed role in sperm maturation processes. Since then, its expression has been detected in many human tissues and through its serine and cysteine protease inhibitory effect, it plays a role in the protection against microbes with proteolytic properties. In contrast to normal expression in healthy tissues, its expression increases significantly in certain cancers of the respiratory system, breast and reproductive organs and is present in high

concentrations in the circulation, which makes it suitable for use as a biomarker in assessing the progression of malignant pathologies (e.g. ovarian cancer, lung cancer). At the same time, in non-malignant pulmonological diseases, such as cystic fibrosis, tuberculosis and interstitial lung disease, serum HE4 level effectively followed the severity of inflammatory processes and was therefore a suitable laboratory parameter for evaluating the clinical condition of such patients. During previous clinical studies, significantly elevated serum HE4 concentration in bacterial ARDS showed a strong relationship with unfavorable outcome. Despite these facts, only two publications are known that investigated the prognostic role of serum HE4 level in COVID-19 disease, therefore, we considered it important to further analyze its role in severe COVID-19.

Development and molecular characteristics of endothelial cell dysfunction in severe COVID-19 disease

In the physiological case, the vascular endothelium performs many functions to maintain the normal homeostasis of the body: they have an influence on hemostasis, oxidative stress, inflammation, vascular permeability, blood flow and blood coagulation processes, as well as ensuring the integrity of the vessel wall. In a resting state, the endothelium can be characterized by anticoagulant, antiadhesive and vasodilatory characteristics.

Endothelium dysfunction is present when one or more of the aforementioned endothelial functions are impaired. Pathogens that have entered the bloodstream or one of their constituents are located on the surface of the endothelial cells and in the intracellular space, through the so-called "pattern recognition receptor" (PRR) molecules, a conserved, non-specific inflammatory cascade mechanism is initiated, which induces the NF-κB and MAPK signal transduction pathways within the cell, and also results in increased expression of IL-6, IL-8, TNF-α, IL-1β, INF-γ and various cell adhesion molecules (e.g. E-selectin, P-selectin, ICAM-1, VCAM-1). Disruption of the endothelium monolayer generates a consequent increase in permeability and enables the procoagulant factors present in the connective tissue, e.g. release of TF, plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF) and urokinase-type plasminogen activator (uPA). In the case of infection caused by SARS-CoV-2, the intercellular connections between endothelial cells and white blood cells, as well as platelets, have been proven to play a prominent role in the development of myocardial infarction (MI), vasculitis, interstitial pneumonitis and ARDS, which are often detected

as complications. In addition to the indirect cell activation mechanisms, the direct infection through ACE2 on the surface of the endothelial cells, the consequent cell activation and damage, and cell apoptosis are also believed to be of great importance.
Atherosclerosis is a characteristic manifestation of chronic endothelial inflammation and dysfunction, which develops because of abnormal functioning of endothelial cells, platelets, macrophages and surrounding smooth muscle cells. Based on recent clinical studies, the molecule Lp-PLA2, also known as "platelet-activating ethyl-hydrolase", plays a key role in the development of the necrotic tissue proliferation that forms the core of the sclerotic plaque. Lp-PLA2 is a protein produced by macrophages that circulates in the blood in a complex with low-density lipoprotein (LDL) and produces proinflammatory mediators from oxidized phospholipids through hydrolysis. The detectable Lp-PLA2 level in the serum proved to be an effective biomarker in the risk estimation of recurrent coronary artery disease and stroke caused by arteriosclerosis, as well as in the evaluation of the procoagulant tendency in lipid metabolism disorders. Despite the involvement of lipid metabolism and the endothelial functional damage in COVID-19 disease, we have a limited number of results on the predictive value of quantitative changes in Lp-PLA2.

A possible difference in the activity of LDH isozymes in COVID-19 disease

The LDH enzyme is an oxidoreductase produced by every cell type, which promotes the reversible conversion of pyruvate to lactate. In case of cell damage, the protein enters the bloodstream, where its activity can increase significantly. The molecule has a tetramer structure and is made up of different combinations of two (M - muscle and H - heart) subunits, which are called isoenzymes and their production shows tissue specificity to a certain extent. A total of five isozymes are distinguished in humans: LDH-1 (subunit 4H) mainly in the heart muscle, LDH-2 (3H1M) in the reticuloendothelial system, LDH-3 (2H2M) in the lung, LDH-4 (1H3M) in the kidney, while LDH-5 (4M) is produced by liver and skeletal muscle cells. In addition to several other routinely measurable laboratory parameters, LDH has also been proven to act as an independent predictive factor in terms of severity in COVID-19 disease and is able to predict severe lung damage and respiratory failure, as well as an unfavorable outcome. Although previous studies have revealed the LDH isoenzyme patterns characteristic of other respiratory diseases, only few clinical results are available for the disease of COVID-19.

2. Objectives

Investigation of soluble ACE2 activity in relation to the outcome of severe and critically ill COVID-19 patients

Based on previous results, we were interested in whether the activity of ACE2 showed a correlation with the clinical severity of COVID-19 and whether there was a visible difference in activity in the case of vascular abnormalities caused by SARS-CoV-2 compared to severe bacterial sepsis. We sought to answer the following questions:

• ACE2 activity of serum samples from patients suffering from severe and critically ill COVID-19 disease was measured and compared to the values in the healthy population and the results of patients treated for severe bacterial sepsis.

• In patients infected with SARS-CoV-2, we determined the activity of soluble ACE2 both before hospitalization and during treatment to evaluate the potential predictive and prognostic value of this biomarker.

• ACE2 activity values were examined in correlation with routine laboratory inflammatory markers, and statistical analysis was used to identify those parameters acting as independent variables.

Examination of serum levels of the pulmonary inflammatory biomarker HE4 in patients with different severities of COVID-19 pneumonia

The examination of HE4 protein expressed by respiratory epithelial cells, seemed obvious in the case of severe SARS-CoV-2 infection, which primarily causes respiratory tract infections. That's why we examined:

• Changes in HE4 serum levels in mild, moderate and critical COVID-19 patients before hospital admission and in follow-up samples in a subgroup of patients.

• HE4 values were correlated with the routine laboratory parameters, the severity of the clinical condition and pulmonological involvement.

• HE4 serum levels measured before hospitalization were correlated with clinical progression and the outcome of the disease to assess possible prognostic significance.

Examination of the concentration of biomarkers characterizing endothelial cell dysfunction and platelet activation in severe COVD-19

Structural and functional damage to the endothelium plays an unquestionable role in the pathomechanism of the COVID-19 disease, which can trigger a cascade-like

inflammatory and coagulation mechanism within the blood vessel. The inflammatory proteins produced at this time and the signaling molecules of secondary platelet activation can be measured in the serum. Consequently,

• We retrospectively examined some cell-derived parameters in the serum samples of survivors suffering from ARDS caused by SARS-CoV-2 infection and patients with an unfavorable outcome. As a control group, we analyzed the samples of patients with mild respiratory infection symptoms but negative SARS-CoV-2 specific RT-PCR.

• To detect endothelial cell dysfunction, the levels of VCAM-1, E-selectin, ACE2 and Lp-PLA2 were determined, while the detection of soluble P-selectin and CD40L characterized platelet activation.

• We established a possible relationship between elevated serum levels of the above biomarkers and the outcome of the COVID-19 disease.

Examination of certain isoenzymes of LDH in mild/moderately severe and severe COVID-19 disease

We explored the changes in the activity of individual isozymes of LDH in COVID-19 pneumonia with different clinical severity. For this purpose,

• We determined total LDH activity in the serum samples of patients with severe and mild/moderately severe COVID-19 requiring hospitalization and correlated our results with the severity of the clinical condition.

• The activity of each LDH isoenzyme individually was measured, and we then looked for a correlation between the isoenzyme activity values and the serum level of other routine laboratory parameters.

• A relationship between the extent of lung involvement and the pattern of differences in LDH isoforms was analyzed.

3. Materials and methods

During the investigations for those publications which form the basis of this doctoral dissertation, we processed serum samples from a large group of COVID-19 patients for the analysis of various biomarkers. To reach our objectives, we classified the patients into different subgroups and followed the outcome of their clinical condition. Of note, many methodological conditions overlapped, so they are not presented individually, but in a unified manner.

Patients and controls

During the second and third "wave" of infection caused by SARS-CoV-2 (from December 2020 to July 2021) mild, severe and critical COVID-19 patients treated at the Department of Infectiology of the University of Debrecen and the Central Intensive Care Unit of the Kenézy Gyula Campus, were enrolled to obtain blood samples. Many patients were treated by the Candidate herself. In addition to this study population, in case of the soluble ACE2 serum activity measurements, we also included samples of a smaller group of COVID-19 patients, who were treated at the National Institute of Hematology and Infectious Diseases at the Budapest Ferenc Jahn Central Hospital. The entire examined patient population included patients between the ages of 18 and 87. Known malignant tumor disease, autoimmune disease, chronic lung disease and pregnancy were treated as general exclusion factors. Acute SARS-CoV-2 infection was confirmed by the RT-PCR method of nasopharyngeal swabs in all patients selected for our study group. After admission to the hospital, severe or critically ill COVID-19 patients were placed in an intensive care unit, while patients requiring oxygen therapy but in a stable condition were placed in an open hospital ward. To judge the severity of the clinical condition in each case, we used the "Sequential Organ Failure Assessment" (SOFA) scoring system. The need for oxygen supplementation and thus indirectly the functional state of the lungs was calculated using the Horowitz index, which can be calculated from the oxygen fraction of the inhaled gas mixture (FiO_2) and the oxygen pressure measured in the patient's arterial blood (PaO_2), the unit of measure being mm Hg (or kPa). The normal value of the Horowitz ratio (also known as the Pa/Fi index) without oxygen support is 350-450 mmHg at normal atmospheric pressure. The difference between the oxygen level measured in inhaled and arterial blood reveals the pulmonary ventilation and perfusion capacity. If either the ventilation of the lung areas

or the delivery of oxygen from the alveoli are disturbed, this is reflected in a decrease in the Pa/Fi ratio. ARDS is the most severe form of respiratory failure, the definition of which is determined by the Berlin criteria laid down in 2012. Based on the Horowitz index, ARDS can be divided into three categories in terms of severity: if the Horowitz index value is between 200-300 mmHg, it is mild, if it is between 100-200 mmHg, it is moderate, and below 100 mmHg indicates severe ARDS. In the case of patients suffering from bacterial sepsis included as a control group in our studies, the diagnostic criteria for sepsis were established based on the criteria system of the American College of Chest Physicians/Society of Critical Care Medicine Consensus criteria.

The standard therapy used in the case of COVID-19 patients requiring hospitalization was carried out in accordance with the Hungarian professional recommendation in force at the time of the study ("Manual for the prevention and therapy of infections caused by the novel coronavirus [SARS-CoV-2] identified in 2020 [COVID-19]"). Oxygen therapy, non-invasive and invasive ventilation were introduced considering the patient's current oxygen demand. We monitored the progression of the clinical condition by regularly re-evaluating the previously presented SOFA score system and Horowitz index. We relied on the data provided by the attending physicians regarding the patients' demographic characteristics, comorbidities, lung involvement visible on chest CT, therapeutic modalities, length of hospitalization, and outcome.

During the soluble ACE2 and HE4 tests, critically ill bacterial septic patients were selected as a control group, those who had a negative SARS-CoV-2 RT-PCR test and the fact of bacterial infection was confirmed by microbiological methods. The diagnosis of sepsis was established according to the current diagnostic criteria of the ACCP/SCCM (American College of Chest Physicians/Society of Critical Care Medicine Consensus). In case in our series of measurements studying the VCAM-1, Lp-PLA2, E-selectin, P-selectin and CD40L markers, we used controls from collected serum samples of SARS-CoV-2 negative patients with mild respiratory symptoms, hospitalized at the Internal Medicine Institute of the University of Debrecen Clinical Center.

At the time of hospital admission, before the start of any therapeutic intervention, anticoagulated (serum) samples were taken from all participants, and peripheral venous blood was taken into Vacutainer® tubes containing sodium citrate, which was supplemented with follow-up samples if possible before discharge or exit. Members of the control groups were sampled during the first 24 hours of hospitalization.

Blood sampling and laboratory analyses

The routine laboratory tests were carried out at the Department of Laboratory Medicine of the Clinical Center of the University of Debrecen. After centrifugation of cell constituents, serum was removed and stored at -70°C until further processing. Anticoagulated blood samples were also centrifuged to store citrated plasma. White blood cell count, platelet count, hemoglobin concentration and mean platelet volume (MPV) were determined under routine conditions on an Advia® 2120 Hematology System (Bayer Diagnostics, Tarrytown, NJ, USA). Detection of C-reactive protein (CRP), procalcitonin (PCT), IL-6, ferritin and cardiac troponin-T (cTnT) concentration was done using by electro-chemiluminescence immunoassay (Cobas® e411, Roche Diagnostics, Mannheim, Germany), while for D-dimer determination, immunoturbidimetric measurements were performed (BCS® XP, Siemens, Munich, Germany). Enzyme activities (aspartate aminotransferase [AST] and alanine aminotransferase [ALT], as well as LDH) and creatinine and urea were determined with a kinetic colorimetric assay using a Cobas® 6000 analyzer (Roche Diagnostics). The value of the estimated glomerular filtration rate (eGFR) was determined based on the "CKD-EPI" (Chronic Kidney Disease Epidemiology Collaboration) equation.

The activity of individual isozymes of LDH was analyzed using gel electrophoresis (LDH Hydragel® 7 kit [Sebia, Norcross, GA, USA]) at pH 8.4 on agarose gel. The isolated isozymes were visualized with a special chromogenic substrate. The amount of formazan precipitate obtained was proportional to the activity of each LDH isozyme. The samples were run on the agarose gel using the semi-automatic HYDRASYS® electrophoresis device (Sebia). The relative amount of each isoform was determined from the dried gel using densitometry. The ratio of LDH isoenzymes was calculated based on the instructions of the manufacturer of the Hydragel®-7 kit. Ms. Erika Dzsudzsák (UD, Department of Laboratory Medicine) provided technical assistance in carrying out these measurements. For the determination of serum ACE2 activity by ELISA, we used a specific fluorescent substrate based on a method presented in a previous publication. We thank Dr. Miklós Fagyas (UD, Institute of Cardiology, Department of Clinical Physiology) for the help in these measurements. Serum HE4 levels were measured using an automated immunoassay (Architect® i1000SR, Abbott Diagnostics, Wiesbaden, Germany). The levels of VCAM-1, E- and P-selectin, and CD40L in the samples were detected using commercially available ELISA kits

according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA), while the detection of Lp-PLA2 - also according to the manufacturer's instructions based on - another automated chemiluminescence immunoassay (Snibe Maglumi® 800, Snibe, Shenzhen, China).

Statistical analyses

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to test the normality of the data. Before the measurements, we determined the size of the smallest sample required to detect significant differences with a statistical power of 90% and an α value of 0.05 using the Intercooled Stata v17 program. Based on previous reports, when measuring the HE4 serum level, the mean value (145.7 pmol/l and 284.0 pmol/l) and the corresponding standard deviation value (118.4 pmol/l) were determined for the groups infected with severe and critical COVID-19, and 201.6 pmol/l) were considered, and the smallest desirable number of samples per subgroup turned out to be 30. In the case of soluble E-selectin determination, based on studies of intensive care unit cohorts with favorable and unfavorable outcomes, median values of 33.7 ng/ml and 60.6 ng/ml and 25.0–45.5 ng/ml and with an IQR of 49.2 -111.6 ng/ml, the minimum sample number was 33. Our results were expressed as a median value (interquartile range, IQR). To compare the samples of two cohorts, we used the Mann-Whitney U test or the $\chi 2$ test, or Fisher's exact test. The comparison of the serum levels of pre-hospitalization and follow-up samples was performed using Wilcoxon's paired test. When comparing more than two groups, we performed the Kruskal-Wallis test with Dunn's post-hoc analysis. The correlation between the examined biomarkers and other clinical and laboratory parameters was demonstrated using the Spearman test. The discriminatory power of the biomarker serum levels measured at hospital admission and the Horowitz index on clinical progression and outcome was evaluated by ROC curve (receiver operating characteristic) analysis by determining the AUC (area under the curve) value. The threshold values were determined based on the maximum of the Youden index. Multiple logistic regression analysis was used to reveal the effect of the tested serum activities on prognosis and clinical outcome, as well as to examine the relationships between demographic and comorbidity characteristics and routine laboratory parameters as dependent variables. A probability level of $p < 0.05$ was considered significant. GraphPad Prism® software (version 6.01, La Jolla, CA, USA) was used for statistical analysis.

4. Results

1) Soluble ACE2 activity in severe and critically ill COVID-19 patients correlates with the severity of the disease and can predict the clinical outcome

Based on our previous study, a significant increase in soluble ACE2 activity was confirmed in patients suffering from severe COVID-19 pneumonia. During our current investigations, we determined ACE2 activity in the serum of a large patient population even before hospitalization and looked for its correlation with the severity of the clinical condition and the unfavorable outcome.

Demographic and anamnestic characteristics of severe and critically ill COVID-19 patients

In this study, 176 COVID-19 positive subjects were recruited to analyze serum ACE2 activity upon hospital admission and during hospital treatment. Based on disease severity, patients were divided into two (critically ill and severe) subgroups as 110 individuals were critically ill patients, while 66 suffered from moderate COVID-19 symptoms. Critically ill patients were older than those with severe disease (median [IQR] 67 [59-76] vs 61 [52-65] years; $P < 0.0001$), while there was no difference in sex between the two cohorts. No difference was observed in the length of hospital stay between the critically ill and severe groups (median [IQR] 10 [5-19] vs 8 [6-12] days; $P = 0.1517$). Mechanical ventilation was applied more frequently in critically ill than in severe COVID-19 subjects (96 vs 6 patients, respectively). Out of 110 critically ill patients, 86 (78.2%) died of COVID-19 disease in contrast to the severe cohort with 3 non-survivors (4.5%) out of 66 patients ($P < 0.0001$). According to the routine laboratory investigation, significantly higher levels of inflammation specific parameters, i.e. CRP, IL-6 and ferritin as well as WBC count, were determined among critically ill than severe COVID-19 conditions. On the other hand, platelet count did not differ between the two cohorts, as no severe thrombocytopenia developed in these subjects. Age- and sex-matched non-COVID-19 severe septic patients showed similar SOFA-scores compared to severe COVID-19 subjects, however, the mortality rate was lower in these non-COVID-19 ICU patients.

The Horowitz index - as an assessment of overall lung function and oxygenation in patients suffering from life-threating pulmonary disorders - was calculated in 106

COVID-19 subjects in whom a greater degree of respiratory distress was indicated. Consequently, critically ill COVID-19 patients (with severe ARDS) showed significantly lower values of Horowitz index (103 [71-160] mmHg) compared to severe cases (147 [89-222] mmHg, $P < 0.0001$), while non-COVID-19 septic subjects had mild/moderate pulmonary disorders based on this index (243 [182-384] mmHg).

Correlation between baseline ACE2 activity and the severity of COVID-19

First, we determined serum ACE2 activity of COVID-19 patients upon hospitalization. ACE2 activity was significantly higher in critically ill (54.4 [36.7-90.8] mU/L) than in severe COVID-19 subjects (34.5 [25.2-48.7] mU/L, $P < 0.0001$) and in non-COVID-19 severe sepsis (40.9 [21.4-65.7] mU/L; $P = 0.0260$) regardless of comorbidities. The direct effect of sex and age on soluble ACE2 level was investigated in the COVID-19 cohort. ACE2 was significantly higher in males than females among critically ill patients ($P = 0.0436$), while this difference could not be seen among severe subjects ($P = 0.2870$). There was a tendency for higher ACE2 activity in relation to increasing age ($P = 0.0134$) regardless of disease severity. However, when this association was separately analyzed within the two severity groups, this trend was not found in either cohort ($P = 0.6841$ and $P = 0.4344$, respectively) suggesting that disease severity but not age modulated serum ACE2 levels. Also, when Spearman's test was used to analyze the correlation between baseline ACE2 and age, no relationship was found ($r = 0.074$, $P = 0.3279$). We then studied the correlation between circulating ACE2 activity and the levels of routinely available laboratory tests suggesting a link between the elevated expression of ACE2 and systemic inflammation causing cardiac, liver and kidney disorders in COVID-19. Based on these results, serum ACE2 activity is strongly associated with the severity of COVID-19, although it is not specific to SARS-CoV-2 infection.

Changes in ACE2 activity among critically ill vs severe COVID-19 clinical conditions

Serum ACE2 activities were determined not only in baseline samples but were followed in a subgroup of recruited critically ill and severe patients to study the kinetics of ACE2 activity depending on COVID-19 severity. We found that, when compared to baseline levels, ACE2 activities were further elevated during the hospital treatment of critically ill patients ($P < 0.0001$), in contrast to severely ill study participants where alterations

did not reach statistical significance during hospital stay (P = 0.0579). Moreover, similarly abnormal ACE2 activity values (P = 0.4165) were found at the admission and subsequent time intervals. Overall, these data imply that ACE2 expression is sustainedly induced under severe COVID-19 conditions, particularly in critically ill patients.

Association between serum ACE2 and the outcome of COVID-19

Serum ACE2 was also analyzed whether this biomarker showed any association with the disease outcome. Accordingly, non-survivors demonstrated significantly higher ACE2 activities (54.6 [37.3-94.7] mU/L) at hospital admission vs survivors (35.6 [25.3-58.5] mU/L (P < 0.0001). Then hypothetical differences in patients' ACE2 activities were further contrasted between initial and follow-up serum samples depending on the outcome on an individual basis. In this context, most (both critically ill and severe) patients who finally died of COVID-19 showed significant ACE2 elevations (P < 0.0001) before death, while no significant change (P = 0.0623) in ACE2 activities were observed before discharge from the hospital in survivors. According to these results, there is a strong association between the level of ACE2 and the clinical consequences of COVID-19 disease.

Suitability of initial ACE2 to predict the severity and outcome of COVID-19

To further investigate initial ACE2 in the prediction of the severity and outcome of COVID-19 disease, ROC-curve analyses were performed. The best discriminative threshold of ACE2 at admission, estimated by Youden-index, was 45.4 mU/L with a sensitivity of 60% and specificity of 71.2% to estimate disease severity at an AUC value of 0.701 (95% CI [0.621-0.781], P < 0.0001). Using the same cut-off value, ACE2 could predict the mortality with a sensitivity of 61.8% and specificity of 65.5% at a similar AUC value of 0.679 (95% CI [0.600-0.759], P < 0.0001).

To test whether ACE2 can independently predict the severity of the disease, a logistic regression analysis was performed. Higher initial ACE2 activity had a significantly higher odds ratio for a more severe outcome (OD: 1.032, 95% CI [1.005-1.061], P = 0.019). Also, ferritin, creatinine, WBC count, lymphocyte count, hemoglobin, and MPV showed statistically significant OD in the prediction of COVID-19 severity.

Taken together, these data indicate that serum ACE2 activity has a capacity to predict the development of adverse clinical events in COVID-19.

Horowitz index in COVID-19 and its relationship with ACE2 levels

As we described above, the values of P/F ratio in patients with critically ill clinical conditions were significantly lower (P = 0.0306) compared to moderate COVID-19 cases. This index was also analyzed regarding the outcome of the disease, and non-survivors had a significantly decreased P/F ratio vs survivors (100.5 [66.3-160] vs 142 [90.7-200.3] mmHg, P = 0.0180). The diagnostic characteristics of P/F ratio was further studied by using ROC-curve analysis. Its best discriminative threshold was 129 mmHg with a sensitivity of 66.7% and specificity of 64.8% for the assessment of disease severity at an AUC value of 0.662 (95% CI [0.522-0.802], P = 0.0312). Using the same cut-off value, ACE2 predicted the outcome of the disease with a sensitivity of 65.8% and specificity of 61.5% at an AUC value of 0.653 (95% CI [0.539-0.767], P = 0.0196). Surprisingly, when ACE2 levels were correlated with the values of Horowitz index, no relationship was observed between the two parameters (in the entire group: r = 0.045, P = 0.6417; within the critically ill subgroup: r = 0.0342, P = 0.7517). According to these preliminary results, Horowitz index is suitable to monitor COVID-19 with lung function disorders although lacking a direct association with the expression of ACE2.

Prediction of 30-day mortality by high initial ACE2 level in patients with COVID-19

Out of the 176 COVID-19 patients, 89 (50.6%) died during the 30-day follow-up. There was a significant difference in baseline ACE2 activity between survivors and non-survivors, and this biomarker predicted the outcome based on ROC analysis. Using the derived cut-off value in Kaplan-Meier analysis, COVID-19 patients with highly elevated ACE2 level (≥ 45.4 mU/L) had a larger risk for 30-day mortality when compared to those with lower ACE2 activity (37.2% vs 64.7%, Log-Rank P < 0.0001). Overall, the measurement of ACE2 at admission can be considered as a useful novel biomarker to predict COVID-19 progression and mortality.

2) Elevated serum levels of HE4 can predict severity of COVID-19 symptoms and disease mortality

Based on the previous promising results of our working group, in the evaluation of the effectiveness of CFTR-specific therapy and the progression of cystic fibrosis, we investigated the amount of HE4 protein detectable in the serum in the case of the COVID-19 disease, which also causes progressive lung damage and requires hospitalization. In the course of our retrospective analysis, we sought to answer the following questions: i) whether the HE4 serum level of hospitalized patients measured before therapy shows a correlation with the severity of their clinical condition; ii) if there is a tendentious change in the serum level of the protein parallel to the progression of the disease; iii) if the relationship of HE4 with the level of classic inflammatory biomarkers and the degree of lung involvement can be demonstrated; and iv) whether the expected outcome of critically ill patients requiring intensive care can be predicted using the initial HE4 serum level.

Baseline clinical features of COVID-19 patients and sepsis controls

During this study, we had blood samples from a total of 99 SARS-CoV-2 positive individuals requiring permanent or temporary hospitalization, confirmed by RT-PCR. Of these, 40 patients in critical condition, another 40 with severe clinical manifestations, and 19 patients with mild symptoms were also able to examine the serum samples obtained during hospital admission and some of the patients during treatment. In terms of age and gender distribution, there were no differences between the subgroups, and we also found no differences in the number of days spent in hospital between the critically ill and severe COVID-19 cohorts (median [IQR], 9.5 [6-18] days vs. 10 [6-13] days). Unsurprisingly, the need for mechanical ventilation was significantly higher in the group of patients in critical condition (37 and 2 patients respectively, $p < 0.0001$). In addition, the rate of adverse outcomes also showed a significant difference (90% vs. 7.5%, $p < 0.0001$). Regarding the general inflammatory serum markers, CRP, PCT, IL-6, ferritin and white blood cell count were significantly higher ($p < 0.001$ and $p < 0.0001$, respectively) in the critical condition COVID-19 group compared to the values of patients in severe condition, while we could not detect a marked difference in the platelet count between the two cohorts. Consistent with the need for mechanical ventilation, the Horowitz index was also significantly lower in the

group of critically ill patients compared to the cohort with severe COVID-19 manifestations (95 [65-139] vs. 154 [100-277], P < 0.001). In addition, there was a significant difference in CRP, ferritin and total LDH activity between the severe and mild COVID-19 subgroups.

In accordance with the need for mechanical ventilation, the Horowitz index was also significantly lower in the group of critically ill patients compared to the group of patients with severe COVID-19 manifestations (95 [65-139] vs. 154 [100-277], p < 0.001). In addition, there was a significant difference in CRP, ferritin and total LDH activity between the severe and mild COVID-19 subgroups.

Thanks to the Intensive Care Department of the University of Debrecen's Institute of Internal Medicine, in addition to the SARS-CoV-2-positive individuals, we had samples of a group of 25 patients suffering from bacterial sepsis matched in age and gender to the critical-condition COVID-19 cohort as a control group. Two-thirds of the septic patients had a positive blood culture (*Escherichia coli*, *Klebsiella pneumoniae, Enterococcus faecalis, Streptococcus pneumoniae*, etc.). Compared to the group of critically ill COVID-19 patients, the SOFA score was lower in their case (12 [10-14] vs. 9 [5-10], p < 0.0001), but significantly higher PCT, white blood cell count and MPV values, and lower platelet count and significantly worse kidney function values were detectable among controls with bacterial sepsis. On the contrary, the rate of unfavorable outcome was lower among them compared to the data of the group of patients with COVID-19 in critical condition.

Increased baseline serum HE4 correlates with COVID-19 disease severity

First, baseline serum HE4 level of each COVID-19 patient was retrospectively measured. Individuals under critically ill COVID-19 clinical conditions had a significantly higher baseline HE4 level than those in the severe or mild COVID-19 cohort (P < 0.0001). Importantly, all HE4 concentrations in the critically ill cohort were above the cut-off value of HE4 used in postmenopausal populations, while 45% of severe COVID-19 subjects showed normal HE4 levels based on this reference value. Due to mild SARS-CoV-2 infection, only 2 patients showed higher HE4 levels than 140 pmol/L. When HE4 results of the critically ill COVID-19 cohort were compared to the non-COVID-19 sepsis patients, similarly high levels were observed but there was no significant difference between the two groups (P = 0.056). Next, the potential effect of age and sex was examined on HE4 serum levels in COVID-19. Sex-related

differences were not found in either COVID-19 cohorts. When the relationship between age and HE4 was investigated, Spearman's test showed a significant correlation between these variables (r = 0.349, P = 0.0015). When COVID-19 patients showing different disease severity were separately investigated, no alteration was seen among critically ill (P = 0.8283) and mild COVID-19 participants (P=0.8015), however, there was still an increasing tendency in HE4 levels among severe subjects by age (P = 0.0285). We also examined the correlation between serum HE4 and the routinely determined laboratory parameters. Our data confirmed that baseline HE4 level demonstrated a moderate but statistically significant positive correlation with general inflammatory markers, such as CRP, IL-6, and WBC counts, certain prognostic markers of COVID-19, *e.g.,* total LDH activity, ferritin, and serum ACE2 activity and finally some important clinical parameters including the SOFA score, and the degree of lung manifestation based on chest CT scanning. In terms of the correlation between the length of hospital stay and HE4, we found an inverse trend between critically ill and severe patients. Under severe clinical conditions, significantly higher HE4 concentrations (P = 0.0007) were measured in those survivors who remained longer at hospital (≥ 10 days), while critically ill COVID-19 with higher HE4 levels (P = 0.1653) died earlier with shorter hospital stay. These results suggest that serum HE4 level before treatment was highly modulated by the severity of the developed COVID-19 clinical status in association with massive systemic inflammation and pulmonary dysfunction.

Kinetics of serum HE4 level in COVID-19 survivors and non-survivors

Recruited COVID-19 subjects were also divided into two other sub-cohorts according to the outcome of the disease, and baseline HE4 values were retrospectively investigated whether this biomarker could imply the outcome of COVID-19 before ICU treatment. Non-survivors had significantly higher HE4 levels (P < 0.0001) at hospital admission than survivors. Serum samples were available in case of 30 COVID-19 patients (22 non-survivors and 8 survivors) to monitor HE4 levels during hospital stay. In response to ICU medication, HE4 concentration tended to decrease, but no significant overall alteration was observed in non-survivors (P = 0.8486). In contrast, there was a significant reduction in serum HE4 before discharge compared to baseline values among those patients who recovered under treatment (P = 0.0039). These results underline that baseline HE4 predicted the outcome of severe COVID-19, and its change during hospitalization also successfully followed the disease progression.

Effectiveness of baseline serum HE4 level to indicate disease outcome and severity of COVID-19

To further evaluate the clinical role of serum HE4 as a new prognostic inflammatory biomarker in COVID-19, we statistically analyzed its diagnostic characteristics to predict the severity and the outcome of this disease. For this purpose, ROC-AUC curve analyses were performed. The best discriminative threshold of HE4 level at admission, estimated by the Youden-index, was 286.3 pmol/L with a sensitivity of 80% and a specificity of 70% to estimate disease severity at an AUC value of 0.816 (95% CI [0.723-0.908], P<0.0001). Moreover, in terms of mortality, the ideal cut-off value of baseline HE4 was 331.7 pmol/L with a sensitivity of 80% and a specificity of 83% to predict the outcome of COVID-19 with an AUC value of 0.874 (95% CI [0.797-0.951], P<0.0001). Based on these results, baseline HE4 was effective to assess the progression of life-threatening COVID-19 disease.

Binary logistic regression analysis was also performed to test whether serum HE4 could independently indicate either the disease severity or the clinical outcome considering other laboratory parameters of these COVID-19 individuals. We found that elevated initial serum HE4 level showed a significantly high adjusted odds ratio (OR) for death (OR: 10.618 [2.331-48.354]; P=0.002). In addition, reduced absolute lymphocyte count (OR: 0.175, 95% CI [0.033-0.925], P=0.040), higher IL-6 (OR: 5.605 [1.516-20.724]; P=0.010), elevated ferritin (OR: 5.911 [1.194-29.259]; P=0.029) and increased WBC count (OR: 5.435 [1.152-25.626]; P=0.032) also had a significant OR value in predicting clinical outcome. To predict the severity of the disease, baseline HE4 level demonstrated a “borderline” significant OR value (OR: 3.642, 95% CI [0.985-13.457], P=0.053), while ferritin (OR: 4.813 [1.237-18.723]; P=0.023) and absolute lymphocyte count (OR: 0.224 [0.055-0.909]; P=0.036) showed a statistically significant OR. Overall, these results support the usefulness of serum HE4 level as a potential prognostic biomarker in case of COVID-19.

Prediction of 30-day mortality by augmented baseline HE4 level in patients with COVID-19

Out of the 80 recruited ICU COVID-19 patients, 35 died during the 30-day follow-up. As we stated above, there was a significant difference in baseline HE4 serum level between COVID-19 survivors and non-survivors, and based on the ROC-AUC curve analysis, this biomarker efficiently estimated the outcome of this disease at the cut-off

value of 331.7 pmol/L. Using this cut-off value in the Kaplan-Meier analysis, COVID-19 patients with highly elevated HE4 levels had a significantly higher risk for 30-day mortality compared to those with lower HE4 concentrations (with a death ratio of 71% vs 19%, respectively, Log rank $P < 0.0001$). When we further followed the clinical status of these patients up to 40 days, 4 more subjects died of COVID-19 in the critically ill population, while no other death case was recorded among those with < 331.7 pmol/L. Taken together, serum HE4 level at ICU admission possessed a capacity to predict the outcome of COVID-19.

3) Examination of serum levels of biomarkers suitable for characterizing endothelial cell dysfunction and platelet activation in severe COVID-19 patients

A deeper understanding of the pathomechanism of SARS-CoV-2 infection and the resulting systemic inflammatory reaction directed attention towards the investigation of generalized, cascade-like endothelial cell damage, the consequent pathological regenerative processes and the mediators involved in them. The rise in the serum level of various cytokines and chemo-adhesive factors released into the circulation by the inflamed endothelium and activated platelets can provide information about the current stage of the disease or can predict further deterioration. During our studies, we examined molecules that play a significant role in endothelial cell dysfunction of other etiologies and in platelet activation in COVID-19, which, according to the literature data available to us so far, may play a significant role in the development of the disease with severe clinical symptoms. We aimed to determine the serum and plasma levels of VCAM-1, ACE2, E-selectin and Lp-PLA2 as biomarkers related to endothelial cell activation, and P-selectin and CD40L as parameters characterizing platelet activation in severe COVID-19 patients. around. We correlated the values of the mentioned biomarkers with each other, then with the routine laboratory and clinical parameters, and finally we tried to determine their predictive role in terms of mortality. Although we have already dealt with the serum activity of soluble ACE2 in a targeted and detailed way in our previous study (see above), in our study to be presented, we primarily tried to shed light on the relationship of the other measured endothelial cells to specific parameters in severe SARS-CoV-2 infection.

Baseline characteristics of COVID-19 patients and clinical controls

In total, 70 severe COVID-19 individuals were involved in this study, who were subgrouped according to the clinical outcome. Regarding age and sex, there was no difference among these study subgroups. The length of hospital stay was similar between non-survivors and survivors (median (IQR), 9.0 (5.5–16) vs. 10 (6.5–13) days). Mechanical ventilation was more frequently applied among those severe COVID-19 patients who died of ARDS vs. survivors (30 out of 33 vs. 2 out of 37 individuals, $P < 0.0001$). In addition, 16 age- and sex-matched non-COVID-19 clinical controls showed significantly lower levels in various laboratory parameters (e.g., CRP, ferritin, LDH) compared to non-survivors and convalescent COVID-19 cases as well. General inflammatory markers, such as CRP, PCT, IL-6, ferritin, and white blood count (WBC), were significantly higher ($P < 0.001$ or $P < 0.0001$, respectively) among COVID-19 non-survivors compared to survivors, while PLT count and MPV did not show a significant difference between the two subgroups. The Horowitz index was calculated in the cases of all patients suffering from COVID-19 and was significantly lower in the cohort of non-survivors vs. survivor patients (95 (65–157) vs. 196 (138–381), $p < 0.001$). Furthermore, lung manifestations were further evaluated via chest CT images, and deceased subjects suffered from more advanced pulmonary involvement compared to convalescent patients (73 (60–80) vs. 50 (30–70) %, $P < 0.05$), which highly contributed to the overall disease outcome. Importantly, no significant difference was observed in pre-COVID-19 comorbidities between the two COVID-19 subcohorts.

Increased baseline VCAM-1 and ACE2 serum levels highly reflect the degree of vascular dysfunction in COVID-19 progression

First, baseline serum levels of VCAM-1, E-selectin, ACE2 and Lp-PLA2 were retrospectively measured to evaluate the degree of vascular disorders with endothelial cell activation and to compare them among study sub-cohorts. Both the soluble VCAM-1 concentration and ACE2 activity showed a statistically significant elevation ($P = 0.0001$ and $P = 0.0038$, respectively) at baseline in non-survivors vs. controls. Moreover, both parameters were significantly altered regarding overall survival. In contrast, E-selectin levels demonstrated only a “borderline” significance ($P = 0.0656$) between deceased cases and controls, while Lp-PLA2 did not alter in COVID-19. In parallel, plasma P-selectin and CD40L levels were analyzed as platelet-activation-

dependent markers and compared to controls; soluble P-selectin was significantly higher (P = 0.0072) in non-survivors, while CD40L values were significantly augmented in both COVID-19 subgroups (P = 0.0012 and P = 0.0022, respectively), but no difference was observed between survivors and non-survivors. Based on these data, VCAM-1 concentration and ACE2 activity at ICU admission effectively reflected the degree of endothelial dysfunction. Next, we statistically investigated the relationship among the selected vascular biomarkers of age, Horowitz index and the routinely determined laboratory parameters using Spearman's test. Age did not alter the level of any of these parameters; however, we excluded its potential influence on current tests via recruiting age-matched patient populations. Importantly, a significant correlation was found between VCAM-1 and the Horowitz index (r = 0.3115), PCT (r = 0.3664), IL-6 (r = 0.4599) and E-selectin (r = 0.3643), while an inverse association was analyzed with renal dysfunction evaluated by GFR (r = −0.5076). We also examined the correlation between serum E-selectin/ACE2 and inflammation-dependent parameters, as well as some prognostic markers (i.e., total LDH activity and ferritin). Our data confirmed that both endothelial biomarkers were significantly correlated with CRP, PCT and IL-6 levels, in addition to LDH activity and ferritin. Surprisingly, serum Lp-PLA2 did not show any relationship with other parameters. When platelet activation markers (i.e., soluble P-selectin and CD40L) were studied for correlations with the endothelial parameters, a moderate but significant link was shown between ACE2 and CD40L (r = 0.2948). These results suggest that, among these biomarkers above, serum VCAM-1 concentrations and ACE2 activity were highly modulated by COVID-19 clinical status in response to vascular inflammation and pulmonary dysfunction, while enhanced platelet reactivity and lipid peroxidation did not substantially affect these vascular markers in our subjects.

Efficacy of baseline serum VCAM-1, E-Selectin and ACE2 levels for early indication of unfavorable disease outcome in severe COVID-19

To further evaluate the clinical usefulness of these serum biomarkers, which showed considerable changes (as described above), as new prognostic biomarkers in COVID-19, we statistically analyzed their diagnostic characteristics to predict the disease outcome using ROC-AUC curve analyses. The best discriminative threshold of VCAM-1 levels at admission, estimated according to the Youden index, was 1420 ng/mL with

a sensitivity of 64% and a specificity of 73% to estimate disease outcome at an AUC value of 0.6855 (95% CI [0.5595–0.8115], P = 0.0077). In the case of serum E-selectin concentration and ACE2 activity, very similar AUC values were determined to distinguish non-survivors from survivors. The ideal cut-off value of baseline E-selectin was 49.3 ng/mL with a sensitivity of 58% and a specificity of 70.3% to predict the outcome of COVID-19 with an AUC value of 0.6523 (95% CI [0.5215–0.7813], P = 0.0286). Finally, serum ACE2 activity had an AUC value of 0.6519 (95% CI [0.5213–0.7826], P = 0.0291) with a 57.6% sensitivity and 70.4% specificity at the cut-off value of 45.2 μU/mL. Based on these results, baseline VCAM-1, E-selectin and ACE2 demonstrated equal effectiveness in assessing the progression of severe COVID-19 disease based on ROC-AUC analysis.

Prediction of 30-Day mortality by elevated baseline VCAM-1 and ACE2 in patients with severe COVID-19

Out of the 70 recruited COVID-19 patients, 33 died during the 30-day follow-up. As we stated before, VCAM-1, E-selectin and ACE2 efficiently estimated the outcome of this disease based on the ROC-AUC curve analysis. Using those cut-off values in the Kaplan–Meier analysis, COVID-19 patients with highly elevated VCAM-1 levels had a significantly higher risk of 30-day mortality compared to those with lower values (with a death ratio of 68% vs. 37%, respectively, Log rank P = 0.0031). Additionally, the baseline ACE2 activity of ≥ 45.2 μU/mL was linked to a higher risk of death with a mortality ratio of 65.7% vs. 40%, respectively, Log rank P = 0.0117). In contrast, increased levels of E-selectin prior to treatment (≥ 49.3 ng/mL) almost significantly (P = 0.0707) predicted mortality in severe COVID-19. Taken together, serum VCAM-1 and ACE2 at ICU admission had a capacity to predict mortality in COVID-19.

4) Profiling of lactate dehydrogenase isoenzymes in COVID-19 disease

Total LDH activity can be considered an independent risk factor in severe cases of coronavirus infection. At the same time, there are no literature data on the kinetics of individual LDH isozymes, even though changes in the activity of individual isozymes may even be specific in severe, multi-organ failure-causing COVID-19 disease.

Baseline clinical characteristics of COVID-19 patients

In this study, 22 consecutive patients (13 males and 9 females) at the age of between (min-max) 27-81 years of age were recruited from March 1 to 14, 2021 at the Clinical Center and Gyula Kenézy Campus, University of Debrecen, Debrecen, Hungary. These subjects suffered from severe (n=14) or moderate (n=8) pneumonia at sampling time point and were confirmed to be positive for COVID-19 disease by reverse transcription polymerase chain reaction (RT-PCR) test of a nasopharyngeal swab. All these patients underwent chest CT scan to evaluate the extent of pulmonary lesions, such as ground-glass opacities and consolidation using a visual scoring system. Also, enrolled subjects suffered from various diseases, such as hypertension, cardiomyopathy, diabetes mellitus, renal disorders, cataract or angina based on their pre-COVID-19 history. Severely ill patients were transferred to the Intensive Care Unit (ICU), while those with moderate symptoms were treated at the Department of Infectious Diseases, Gyula Kenézy Campus, University of Debrecen, Debrecen, Hungary. Despite ICU treatment all severe subjects died of COVID-19 within 28 days of the initiation of the disease, while patients in moderate clinical status were effectively treated and survived.

Changes in serum total LDH activity and levels of routine laboratory markers in severe and mild/moderate COVID-19 patients

Based on routine laboratory tests, inflammatory clinical conditions were indicated by elevated WBC count, serum CRP and ferritin levels. Importantly, based on its upper reference limit (URL), i.e. 220 U/L, total LDH activity in sera was higher than normal in all recruited COVID-19 patients within the range of 272-2141 U/L. Moreover, total LDH activity was significantly higher in severe compared to non-severe COVID-19 patients (median [IQR] 947.5 [704.3-1307.0] vs 391.5 [331.8-895.8] U/L, P = 0.016). Although these subjects suffered from various comorbidities in the pre-COVID-19 era,

these conditions did not substantially modulate LDH activities during COVID-19 disease.

The activity pattern of individual LDH isoenzymes in pneumonia caused by COVID-19 disease

To further investigate the background of high total LDH activity, gel electrophoresis was performed to determine the relative abundance and to quantify the calculated activity of LDH isoenzymes. According to the subsequent densitometry analysis, LDH isoenzymes with increased activity had no universal pattern in COVID-19. Out of 22 subjects, nine patients showed a larger ratio of mid-zone fractions, i.e. increased LDH3 with or without LDH-4 or LDH-2, while single elevated LDH-2 activity was seen in case of three patients. Also, there were six individuals with increased LDH-5 activity with or without LDH-4 and two patients had higher LDH-1 level. In contrast, two persons did not show an altered ratio of LDH isoenzymes despite high total LDH activity. Overall, there was no typical profile of LDH isoenzymes in COVID-19 pneumonia.

In contrast, when we statistically correlated the measured activity of total LDH with the calculated activity of each individual isoenzyme, a significant relationship was observed between total LDH with LDH-3 activity ($r = 0.765$, $P = 0.0001$) and LDH-4 activity ($r = 0.783$, $P = 0.0001$), while no association was found with other isoenzymes. The direct link between calculated LDH-3 and LDH-4 activity and other laboratory parameters typically altered in COVID-19 disease was also studied, and a modest but statistically significant association was demonstrated in case of both isoenzymes only with serum ferritin ($r= 0.437$, $P = 0.042$; $r = 0.505$, $P = 0.016$, respectively) .

Lung parenchymal affection (expressed in %) caused by SARS-CoV-2 infection was variable among recruited patients with different disease severity based on chest CT examination. The extent of pulmonary lesions was significantly larger in non-survivors with severe symptoms compared to survivors having only moderate alterations (70 [50-76] vs 15 [50-76] %, $P = 0.003$). When the relationship between CT findings and abnormal relative percentage of LDH isoenzymes was studied, a larger ratio of mid-zone fractions was observed in patients suffered from ≥ 50% pulmonary parenchymal involvement. On the other hand, elevated relative abundance of LDH-2 alone was present at moderate (< 20%) parenchymal extension. Pneumonia and COVID-19

related severe liver failure together resulted in augmented LDH-5 ratio (n=6), while intravascular hemolysis in two critically ill patients showed high LDH-1 level (n=2). Based on these data, the severity of pulmonary affection was strongly related to abnormal relative abundance of LDH isoenzymes which belong to the mid-zone fractions, however, the manifestation of other comorbidities causing the release of other LDH isoenzyme(s) modified the overall results of LDH isoenzyme ratio. Finally, regarding the clinical outcome, significantly larger activity values of LDH-3 (241.0 [127.7-299.0] vs 83.7 [63.0-200.9] U/L, P = 0.043) and LDH-4 (106.4 [85.5-182.7] vs 33.0 [20.9-69.4] U/L, P = 0.034) were seen in non-survivors vs survivors. Furthermore, the relative abundance of LDH-4 (P = 0.026) but not LDH-3 (P = 0.368) was higher in patients with poor outcome than recovered subjects.

5. Discussion

The disease COVID-19 caused by SARS-CoV-2 is a serious, even life-threatening infectious disease that mainly affects the lungs, but in many cases other organ systems in parallel. Based on the suspicion raised by the symptoms, the diagnosis is based on the detection of viral RNA from the patient's nose-throat discharge sample using the RT-PCR technique. Certain demographic factors, such as advanced age, diabetes, chronic lung and cardiovascular disease, chronic renal failure, presence of malignant disease, immunosuppressive status, obesity and sickle cell anemia are independent predictive factors of severe and unfavorable outcome for COVID-19 disease. The clinical and laboratory parameters detected in the early stage of infection can also serve as a reference point in the recognition of patients with a higher risk. Hypoxia detected at the time of hospital admission is an important independent risk factor for an unfavorable outcome. In a previous clinical trial, based on data from 140 severe patients with COVID-19 pneumonia, a value of peripheral arterial saturation (SpO_2) higher than 90% was able to predict survival with 84% sensitivity and 97% specificity. The PaO_2/FiO_2 ratio or Horowitz index is a widely accepted parameter in clinical practice for characterizing hypoxemia and the efficiency of gas exchange in the lungs. One of the criteria for the severe respiratory failure caused by COVID-19 is a PaO_2/FiO_2 ratio below 300 mmHg. Based on retrospective analyses, the ratio determined at the

beginning of the course of the disease separated mild cases from those requiring hospitalization with a good predictive value, and it may also have an influence on the length of hospitalization, and patients with a value below 300 mmHg had up to three times greater chance of an unfavorable outcome. It is a generally accepted practice that prior to each hospital treatment, a peripheral venous blood sample is taken from which the routine laboratory parameters are determined. Since the outbreak of the pandemic, a large amount of data has been collected, and several meta-analyses thoroughly examined the efficacy of these parameters to predict the outcome. For instance, the initial lymphopenia showed a substantial progression with the severity of the disease. The neutrophil/lymphocyte ratio (NLR) was increased when the clinical condition worsened and thus was a predictive factor of the unfavorable outcome. A continuous decrease in the proportion of eosinophils also indicated a negative prognosis. There are conflicting results regarding changes in platelet count: initial studies reported severe thrombocytopenia among critically ill patients and showed a correlation between the degree of platelet count reduction and the likelihood of death. Although other authors found no significant difference in platelet count among COVID-19 patients with ARDS. Among the biochemical factors, LDH, AST, ALT, creatinine, total creatinine kinase (CK), cTnI and NT-proBNP were detected in significantly higher concentrations in the samples of patients with an unfavorable outcome than in those who survived. Similarly, some acute phase proteins (e.g. CRP, PCT, ferritin, serum amyloid-A) were present in significantly higher quantities in populations of severe COVID-19 patients than in those with milder symptoms, and at the same time predicted an unfavorable prognosis. D-dimer above 1 μg/ml was found to be an independent predictive factor of adverse prognosis among hospitalized patients, and it also predicted a higher probability of thromboembolic complications, critical clinical status and death. It would be essential to identify patients prone to an unfavorable prognosis at the first medical examination, so that these patients can receive aggressive supportive therapy and closer monitoring.

The aim of our study series was to verify the increased expression of molecules and mediators present in detectable amounts in the serum and presumably involved in the progression of the SARS-CoV-2 infection, and to detect a relationship between the severity of the clinical symptoms and the serum levels of the examined biomarkers. Furthermore, our goal was to search for serum markers that are highly likely to predict an unfavorable outcome even before the start of hospital treatment.

The ACE2 glycoprotein expressed on the cell surface is one of the most important players in the infection process of human host cells. The ACE2 expressed on the surface of epithelial cells and endothelial cells is used as a receptor by the S1 and S2 surface proteins of the virus, and this bond causes the endocytosis of the virus. Through a kind of protective mechanism to prevent internalization, the binding of SARS-CoV-2 to the detachment of ACE2, the so-called it leads to "shedding", thereby increasing the level and activity of circulating ACE2. In COVID-19, several research groups investigated the amount of soluble ACE2 with different results. Our working group was the first to demonstrate significantly increased serum activity of soluble ACE2 during a case study of a critically ill COVID-19 patient.

During our investigations, we came to the conclusion that significantly higher ACE2 activity was detected in the serum samples of the critically ill COVID-19 patients compared to the values of a severe COVID-19 cohort and a severe septic control group with non-COVID-19 etiology - regardless of comorbidities. In the case of the examined COVID-19 patients, we measured even higher ACE2 activity values among men. Although 80.9% of our critically ill COVID-19 patients suffered from hypertension, abnormal ACE2 activity values can primarily be attributed to severe SASR-CoV-2 infection. Compared to the entire studied population, regardless of the severity of the symptoms, the older patients showed higher ACE2 activity, but we could not verify the effect of age on the ACE2 level in the individual study subgroups using Spearman analysis. Hence, we concluded that the cause of the elevated ACE2 activity is mainly found in cardiovascular diseases that occur in a higher proportion in advanced age and in the more severe course of COVID-19 disease, which is more often seen in the elderly. Based on the follow-up tests obtained during hospitalization, we detected a further significant increase in the serum level of the critically ill COVID-19 patients compared to the initial soluble ACE2 level compared to the serum levels of patients with severe pneumonia where it did not change significantly during treatment. It is important to highlight that in the case of the COVID-19 patients who required hospitalization, the soluble ACE2 levels before treatment remained significantly and permanently elevated or even showed a further increase, and we did not detect a significantly decreasing trend in the recovering patients either. In patients who died due to COVID-19, initial ACE2 activity was significantly higher that showed a further significant increase in the later stages of the treatment - typically shortly before exit. Since the greatest clinical significance was expected from the effect of the soluble ACE2 serum level, which can

be measured at the onset of the initial symptoms, we investigated the prognostic potential of the biomarker in terms of severity and outcome with statistical analysis. With ROC curve analysis, with an initial ACE2 serum level of 45.4 mU/l, 60% sensitivity and 71.2% specificity, but at an AUC value of 0.701, the course of the disease is severe, and using the same "cut-off" value, 61.8% sensitivity and with a specificity of 65.5%, the unfavorable outcome could also be predicted with a similarly high AUC value (0.679). Based on logistic regression analysis, in addition to ferritin, creatinine, white blood cell and lymphocyte count, hemoglobin, and MPV, baseline ACE2 activity was shown to be an independent predictive factor in our study group (OR: 1.032, $p = 0.019$).

In the case of the Horowitz index, in accordance with our expectations, among individuals with an unfavorable outcome, we calculated a significantly lower initial PaO_2/FiO_2 ratio compared to the values of the survivors, the discriminative value of which was determined by the Youden index at 129 mmHg. For predicting the severity of the disease, 66.7% sensitivity, 64.8% specificity and an AUC value of 0.662 were found, while predicting the outcome was performed with 65.8% sensitivity and 61.5% specificity and with an AUC value of 0.653. Even though the available literature data suggest that a significant part of the high soluble ACE2 originates from the surface of the epithelial cells of the infected lungs, during further statistical analysis, the statistical correlation between the soluble ACE2 and the Horowitz index, which consequently worsens with pulmonary functional impairment, was not observed. During the 30-day follow-up period after hospital admission, 89 out of 176 COVID-19 patients selected for our study died. Based on the Kaplan-Meier curve analysis performed with the initial 45.4 mU/l ACE2 serum level as a discriminative value, a significantly higher 30-day mortality probability was shown for patients with a serum level above the threshold (37.2% and 64.7%, Log-Rank $p < 0$ .0001). Based on the tests performed, the serum ACE2 activity, which can be measured before treatment, proved to be a new effective biomarker in the case of COVID-19 patients in predicting both the course of the disease and the expected outcome.

HE4 is primarily expressed in significant amounts on the surface of the epithelial cells of the upper and lower respiratory tract, and its serum level rises significantly and consistently in some oncological pathologies affecting glandular organs. In addition, increased HE4 serum level can be considered as a potential biomarker in chronic renal

failure, and in progressive interstitial pulmonary fibrosis. Previous studies of our working group confirmed a significantly elevated HE4 serum level in cystic fibrosis, the changes of which showed a close correlation with the progression of the disease, respiratory function parameters and the effectiveness of CFTR-specific drug treatment. Based on recently published reports, in ARDS associated with severe bacterial sepsis, HE4 serum level was an independent predictive factor of adverse outcome. Even though, the role of HE4 protein in humoral immunity against pathogens introduced through the respiratory tract is assumed, modest literature data was available regarding changes in HE4 serum levels during SARS-CoV-2 infection.

In our study, in addition to the serum samples of mild, severe and critically ill COVID-19 patients, we analyzed HE4 serum levels measured at hospital admission in the samples of people suffering from bacterial sepsis as a control group. Significantly higher than normal HE4 serum levels were detected in both COVID-19 patients with serious lung involvement and control patients with bacterial sepsis. In the entire study population, HE4 level showed a correlation with age, which is in line with previous literature data, although we could no longer detect this tendency in subgroups differentiated according to severity. Several laboratory parameters (e.g. soluble ACE2, IL-6, CRP, ferritin, total LDH, white blood cell count, etc.) and parameters characterizing the clinical condition (i.e. SOFA-score, degree of lung involvement visible on chest CT) showed a correlation with the initial HE4 serum levels. In addition, regarding HE4 and the number of days in hospital, there was a direct positive association in severe COVID-19 patients, but an inverse relation was shown in the case of patients in critical condition, who usually exited shortly after admission. Considering the above results, the initial HE4 level can most likely indicate severe lung destruction and the extent of generalized inflammation at the time of the first symptoms. During a retrospective analysis, we were able to measure significantly higher initial values in patients who later died, which did not show any significant changes during the treatment until death occurred. On the other hand, in the case of those who left cured after the treatment, HE4 concentration of the follow-up serum samples showed a significant decrease compared to the values before the treatment ($p = 0.0039$). With ROC-AUC analysis, with a serum HE4 "cut-off" value of 286.3 pmol/l, the severe course of the disease could be predicted with an exceptionally good AUC value of 0.816 with 80% sensitivity and 70% specificity, and 331.7 pmol/l baseline HE4 level with 80% sensitivity and 83% specificity at 0.874 AUC value also predicted the unfavorable

outcome. To determine the independent predictive value of this biomarker, a logistic regression analysis was performed, and HE4 serum level was associated with a high odds ratio for an unfavorable outcome (OR: 10.618) along with other laboratory parameters with a previously proven prognostic role (i.e. absolute lymphocyte count, total white blood cell count, IL-6, ferritin). In addition, in terms of the expected severity, a much more modest but still significant correlation was shown in connection with the HE4 level. During the 30-day follow-up period, the probability of mortality in patients with serum levels below and above the 331.7 pmol/l discriminatory threshold mentioned above showed a significant difference (71% and 19% mortality, Log rank $p < 0.0001$). Extending the follow-up period to 40 days, the statistical difference between the two groups was even more substantial (81% vs. 19%). Considering these above, serum HE4 measured at the beginning of hospitalization can be an important prognostic factor both in predicting the expected course and the outcome of COVID-19 disease as well as in the early risk stratification of such patients.

The vascular endothelium plays an active, autocrine and paracrine role in many local and systemic physiological processes, and its flawless functioning is essential for the dynamic optimization of tissue blood supply, the balance of hemostasis and coagulation processes. Numerous research data and clinical observations support the importance of endothelial injury after severe SARS-CoV-2 infection which may manifest in blood supply disorder and blood vessel occlusion causing myocardial infarction, vasculitis, interstitial pneumonitis or even ARDS, which often occurs in the course of the disease of COVID-19. We examined the serum levels of molecules associated with the activation of endothelial cells in severe and critically ill COVID-19 patients to explore their kinetics in relation to the course of the disease, the severity of the disease, and the outcome. We extended our retrospective analysis to critically ill patients forming two subgroups according to favorable and unfavorable outcomes.

The selectins are glycoproteins responsible for the recruitment of white blood cells. E-selectin is an adhesion molecule that enters the circulation when endothelial cells are activated and represents one of the most sensitive biomarkers of endothelial cell activation. P-selectin is located in the Weibel-Palade bodies of endothelial cells and in the intracellular α-granules of platelets, and its expression increases as a result of a trigger upon the activation of these cell types. Based on a meta-analysis summarizing the data of 7,668 patients, in addition to several other biomarkers associated with endothelial cell dysfunction, baseline serum levels of soluble E- and P-selectin were

also higher among SARS-CoV-2 infected patients with an unfavorable outcome. According to a recently published study, significantly higher levels of E-selectin (≥ 32.5 ng/ml) and P-selectin (≥ 3.2 ng/ml) at hospital admission were independent predictors of thromboembolic events during hospitalization, which were associated with severe endothelial dysfunction that supports the existence of a prothrombotic clinical condition. Pre-treatment serum E-selectin level of COVID-19 patients requiring intensive care unit reliably predicted adverse outcome at a remarkable AUC value of 0.88 with 100% sensitivity and 75% specificity. In contrast to our expectations, we could detect only a modest but significant difference between baseline E-selectin values of survivors and deceased COVID-19 patients. Although the initial E-selectin serum level of 49.3 ng/ml showed an AUC value of 0.6523 during the examination of the effect on the outcome, we failed to detect a significant difference between those affected with levels below and above the discriminatory value using Kaplan-Meier survival curve analysis.

Lp-PLA2 is a molecule produced by macrophages that circulates mainly in association with LDL. Through the hydrolysis of oxidized lipoproteins, Lp-PLA2 plays a role in the formation of proinflammatory mediators, which are of particular importance in the activation of endothelial cells and the formation of the necrotic core of arteriosclerotic plaques. Measurement of Lp-PLA2 serum level as a predictive factor is also included in European and American recommendations for the prediction of stroke and recurrent cardiac events. During previous investigations, a strong correlation was shown between the APACHE II score used for the risk classification of critically ill patients and the Lp-PLA2 serum level. In our present study, no significant difference was detected in the baseline Lp-PLA2 serum level of the COVID-19 patients with a favorable and unfavorable outcome. Based on our results, the level of Lp-PLA2 did not correlate with the inflammatory parameters and did not show typical kinetic parallel to the progression either. The background of our results may be the change in the serum level of Lp-PLA2, which is predominantly associated with arteriosclerosis, and the endothelial damage caused by COVID-19, which has a negligible effect on this molecule.

Among the various cell adhesion molecules produced by endothelial cells, VCAM-1 showed significantly elevated serum levels in several preliminary clinical studies in severe COVID-19 patients, either compared to a healthy population or to those infected with SARS-CoV-2 showing only mild symptoms. A study of hospitalized patients with

mild and severe respiratory failure reported higher VCAM-1 levels in patients with an unfavorable outcome. According to further analyses, the serum VCAM-1 level measured before hospitalization also showed a correlation with the probability of 30-day mortality.

The cell-cell interaction between platelets and neutrophils is a sensitive marker of platelet activation. Baseline P-selectin concentration showed a strong correlation with adverse outcome and the frequency of thromboembolic events during treatment. Significantly higher initial P-selectin levels were found in the case of critically ill patients who later died, while only a moderate increase was detectable in the case of recovered patients.

Finally, we analyzed the possible correlations between individual LDH isoenzyme activities and clinical symptoms in the case of 22 patients with mild/moderately severe and severe pneumonia caused by COVID-19. Several previous studies have confirmed that the initial total LDH activity is an independent prognostic factor in the severity of the clinical manifestation and the expected outcome of the COVID-19 disease. The five types of LDH isoenzymes found in the human body show relatively high organ specificity. Accordingly, the isozyme profile behind the total LDH activity was examined. Although we did not experience an exclusive increase in activity typical of COVID-19 in the case of any of the isoforms, the sera of nine of the 22 patients tested showed increased "mid-field activity" during the gel electrophoresis performed, i.e. LDH-3 and/or LDH-2 and LDH-4. A relatively high activity of LDH-4 isoforms was detectable. Furthermore, the LDH-3 and LDH-4 activity calculated based on densitometric analysis showed a significant correlation with the total LDH activity measured from the serum and with the serum ferritin level. In the case of our study, during the chest CT examination, a stronger signal could be detected regarding the activity of some LDH isoenzymes in patients with more than 50% lung involvement, and an increase in the relative activity of the LDH-2 isoenzyme appeared with a mild, lower than 20% pulmonary dissemination. Consequently, the increase in LDH-3 and LDH-4 activity detectable in the serum may be strongly related to the involvement of the lower respiratory tract by COVID-19. However, we must consider the influence of additional organ damage caused by SARS-CoV-2 infection on LDH isoenzymes: LDH-5 elevation can be associated with cell death caused by both pneumonia and liver failure, possible intravascular hemolysis occurring as a consequence of immune-thrombotic processes leading to increased LDH-1 activity. Overall, our results suggest

that although a typical LDH isozyme "pattern" for COVID-19 is probably not detectable, elevated LDH-3 and LDH-4 isozyme activity may show a close correlation with an unfavorable outcome, just as previous studies have already confirmed in the case of total LDH.

6. Summary

COVID-19 disease caused by the SARS-CoV-2 virus, is an infectious clinical condition with high contagiousness that usually results in mild respiratory symptoms. However, a few percentages of COVID-19 patients develop severe pneumonia with multiorgan failure that requires hospital treatment and may lead to death. The exact mechanism of this infection is still unknown, and vaccinations based on various mechanisms have become available to avoid infection in addition to general hygiene rules. The pathomechanism of COVID-19 is most likely based on the direct and indirect endothelial activation caused by SARS-CoV-2, the consequent hyperinflammation and secondary intravascular immune-thrombotic events which typically lead to damage and loss of function of the lungs and in some cases, other vital organs.

In the initial stage of the COVID-19 pandemic, there was no test or set of clinical symptoms that could undoubtedly differentiate those patients with a higher risk for a more severe course or an unfavorable outcome of the disease. The aim of our study series was to identify some new biomarkers in the serum/plasma that can reliably predict disease course and mortality in the initial phase of severe COVID-19.

Based on our results, elevated serum ACE2 activity and augmented HE4 level at hospital admission reliably predicted the subsequent severe course of the disease and the unfavorable outcome. Among the classic biomarkers that can demonstrate endothelial cell activation and platelet dysfunction, early serum levels of VCAM-1 and E-selectin showed a strong correlation with a higher mortality risk. In addition, during the examination of the background of total LDH activity, which had been earlier known as a prognostic role in severe COVID-19 disease, we could not detect a typical isoenzyme activity pattern characteristic to severe SARS-CoV-2 infection.

Overall, we conclude that elevated soluble ACE2 and HE4 levels detected in the early stages of the COVID-19 disease reliably stratify those patients in whom a more

severe disease course can be expected. In case of these patients, a closer clinical observation is warranted due to a higher risk of critical respiratory failure, and there is a clinical need for invasive ventilation and possible thromboembolic complications. The proactive clinical approach used in the treatment of the high-risk patient group with early supportive therapy according to the early detection of possible deterioration, can prevent the development of critical complications or irreversible damage, which may lead to the death of these patients.

7. New scientific results of the thesis

5) In severe and critically ill COVID-19 patients, ACE2 serum activity before hospitalization shows a strong correlation with disease prognosis and mortality.

6) The baseline serum HE4 level significantly increases in COVID-19 pneumonia, it can distinguish critically ill and severe COVID-19 patients from each other and predicts the risk of early death.

7) Soluble VCAM-1 and E-selectin levels indicating increased endothelial cell activation, reliably predict the adverse outcomes in critically ill COVID-19 patients.

8) Although no clear COVID-19-specific "pattern" can be detected in the serum activity of individual LDH isoenzymes, a significant difference in LDH-3 and LDH-4 activity can be seen between COVID-19 survivors and non-survivors.

Keywords: COVID-19, SARS-CoV-2, Pneumonia, ARDS, Endothelial disfunction, Inflammation, ACE2, RAAS, HE4, VCAM-1, E-selectin, P-selectin, CD40L, Platelet activation, Lp-PLA2, LDH isoenzymes

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9. References

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List of publications related to the dissertation

1. **Sütő, R.**, Pócsi, M., Fagyas, M., Kalina, E., Fejes, Z., Szentkereszty, Z., Kappelmayer, J., Nagy, B. Jr.: Comparison of Different Vascular Biomarkers for Predicting In-Hospital Mortality in Severe SARS-CoV-2 Infection.
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5. Csoma, E., Nagy, K. Á., **Sütő, R.**, Szakács, S. E., Pócsi, M., Nagy, A. C., Bíró, K., Kappelmayer, J., Nagy, B. Jr.: Evaluation of the diagnostic performance of two automated SARS-CoV-2 neutralization immunoassays following two doses of mRNA, adenoviral vector, and inactivated whole-virus vaccinations in COVID-19 naïve subjects.
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