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Neopterin and kynurenine in serum and urine as prognostic biomarkers in hospitalized patients with delta and omicron variant SARS-CoV-2 infection

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Abstract

Objectives: Currently, no biomarker or scoring system could clearly identify patients at risk of progression to a severe coronavirus disease (COVID)-19. Even in patients with known risk factors, the fulminant course cannot be predicted with certainty. Analysis of commonly determined clinical parameters (frailty score, age, or body mass index) together with routine biomarkers of host response (C-reactive protein and viral nucleocapsid protein) in combination with new biomarkers neopterin, kynurenine, and tryptophan, could aid in predicting the patient outcome.

Methods: In 2021 and 2022, urine and serum samples were prospectively collected on 1st to 4th day after hospital admission in 108 consecutive COVID-19 patients hospitalized at the University Hospital Hradec Králové, Czech Republic. Delta and omicron virus variants were studied. Neopterin, kynurenine and tryptophan were determined by liquid chromatography.

Results: A significant correlation was observed between urinary and serum biomarker concentrations. Urinary and serum neopterin, kynurenine and kynurenine/tryptophan ratio were significantly ($p \leq 0.05$) higher in patients who subsequently needed oxygen therapy vs. patients without oxygen therapy. These parameters were also significantly increased in patients who died during the hospitalization compared to survivors. Complex equations have been derived using the investigated biomarkers and other clinical or laboratory parameters to predict the risk of subsequent oxygen therapy or death during hospitalization.

Conclusions: Present data demonstrate that neopterin, kynurenine and kynurenine/tryptophan ratio in the serum or in the urine represent promising biomarkers in the management of COVID-19 that may help to guide important therapeutic decisions.

Keywords: COVID-19; HPLC; kynurenine; neopterin; prognosis; urine.

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Introduction

Coronavirus disease 2019 (COVID-19) is an acute viral respiratory infection first described at the end of 2019 in the Chinese city of Wuhan that is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an RNA virus belonging to the betacoronaviruses. In the subsequent months, SARS-CoV-2 spread rapidly throughout the world leading to a global pandemic with profound impact not only on health care systems, but also on the economy and the society. In most cases, SARS-CoV-2 infection is asymptomatic or accompanied only by mild symptoms. The virus spreads through droplets, and after the binding of the viral spike protein to angiotensin-converting enzyme 2 (ACE2) on the cell membrane enters cells and replicates. Cell membrane

ACE2 is present in practically all tissues, being most abundantly expressed in the lung or intestinal epithelial cells and capillary endothelium [1]. Flu-like symptoms characterize the initial phase of COVID-19, which may, in some cases, lead to a cytokine storm. A severe course with the development of acute respiratory distress syndrome can result in long-term lung damage or even death.

Neopterin, a well-established immune activation biomarker is produced from guanosine triphosphate. Serum or urinary neopterin concentrations reflect the activation of the immune system and predict outcomes across a range of different disorders, including viral infections [2]. Monocyte-derived macrophages produce neopterin after stimulation with interferon-gamma that also induces the conversion of tryptophan to N-formylkynurenine and subsequently to more stable kynurenine by indoleamine 2,3-dioxygenase (IDO). Interferon-gamma production after immune system activation during viral infection may lead to elevated levels of neopterin and degradation of tryptophan to kynurenine, resulting in an increased kynurenine/tryptophan ratio. The inflammatory response during COVID-19 results in monocyte/macrophage activation and the release of pro-inflammatory cytokines (Figure 1). Numerous studies show a positive correlation between inflammatory biomarkers such as C-reactive protein (CRP) or interleukin-6 with the disease severity [3, 4]. Recently, some researchers have focused on exploring neopterin and kynurenine/tryptophan ratio as a reliable inflammatory biomarker which could give similar or superior information compared to routinely used biomarkers [5]. This theory could be supported by the study of Zheng in 2005 et al. [6], in patients with SARS disease, where serum neopterin levels were significantly elevated in comparison to the control group, while CRP levels did not show any differences. The correlation of neopterin concentration with the disease severity was also confirmed in COVID-19 patients in other recent studies [7] indicating that neopterin might be used as a biomarker for the prediction of disease severity in COVID-19 patients. IDO activation assessed by determining kynurenine/tryptophan ratio and potential correlation to neopterin concentration was also investigated in COVID-19 [8–10].

Bellmann-Weiler et al. reported that serum neopterin above 45 nmol/L is associated with a 14-fold higher risk of Intensive care unit (ICU) admission during the hospital stay and a 16-fold increased the need for mechanical ventilation [11].

All these studies involved relatively small cohorts of patients infected with delta or earlier (i.e. beta, alpha, or gamma) SARS-CoV-2 variants and used enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) [10–12], which are commonly used techniques in clinical practice.

The correlation between RIA and ELISA is fair, but RIA requires relatively expensive equipment and involves radionuclides use. Immunoassays generally suffer from cross reactions leading to false positive and false negative results. Compared to separation techniques, a significant disadvantage is that only one analyte may be determined at a time.

The most suitable sensitive and selective separation method for the determination of neopterin, kynurenine, and tryptophan is high performance liquid chromatography

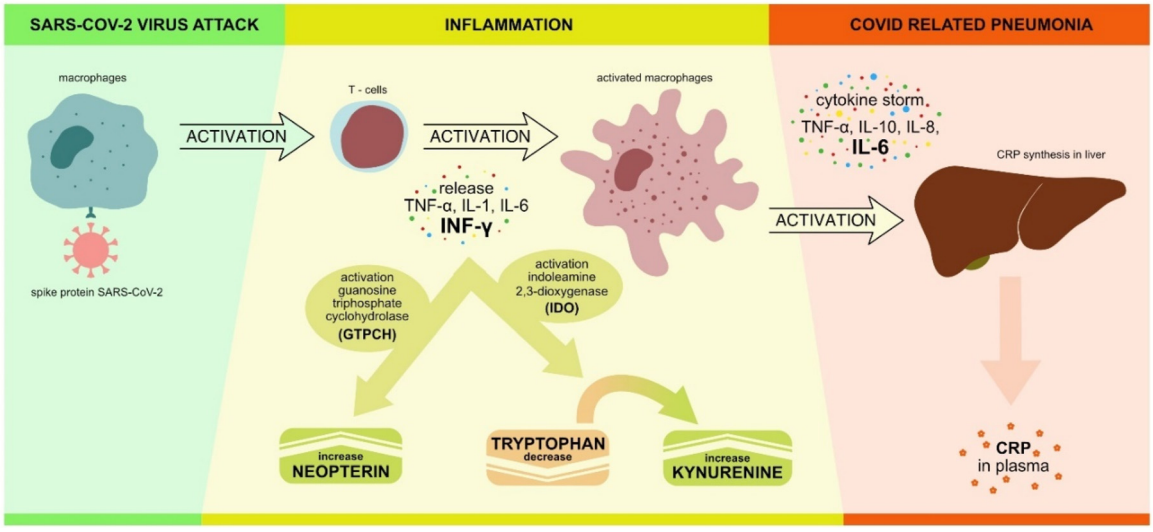

Figure 1: The inflammatory response during COVID-19 disease. IL – interleukin, CRP – C-reactive protein, TNF-α – tumour necrosis factor alpha, INF-γ interferon gamma.

(HPLC) with fluorescence detection (FLD) (neopterin) or mass spectrometry (MS) (kynurenine and tryptophan) detection using internal standardization for compensation of pre-analytical phase and matrix effect in MS detection. The principal advantage of this technique is the ability to determine a wide range of substances in a single analysis, which is not possible with conventional immunochemical methods.

In our laboratory, we have developed HPLC-FLD/MSMS methods for the determination of these analytes in various biological fluids, including saliva, urine, ascites, exudate, or amniotic fluid [13–15], and we have many years of experience with clinical studies using these methods in patients with various disorders [16].

The principal aims of the present study included:

(1) Evaluation of the correlation between urinary and serum biomarkers exploring the possible use of urine as a noninvasively obtained sample matrix,
(2) Exploration of the potential use of neopterin, kynurenine and tryptophan as predictors of disease severity in the entire cohort of hospitalized COVID-19 patients and, separately, in patients infected with delta and omicron virus variant,
(3) Assessment of the potential role of these urinary and serum biomarkers as predictors of the disease prognosis.

Materials and methods

Samples were obtained from consecutive patients 18 years or older hospitalized at University Hospital Hradec Králové, Czech Republic between November 2021 and April 2022 with COVID-19. The patient characteristics are shown in Table 1.

The study protocol was approved by the Institutional Ethics Committees (No 202011P04), and all patients signed informed consent.

Serum and urine samples were obtained on 1st to 4th day after hospital admission. Samples were used to measure neopterin, kynurenine, tryptophan, and urinary creatinine to correct for urine dilution. Serum and urinary samples were protected against light and transported to the laboratory immediately after collection. Serum neopterin, kynurenine, and tryptophan were measured using HPLC-FLD/PDA method [17], and urinary neopterin, kynurenine, tryptophan and creatinine were determined using HPLC-FLD/PDA [18] and UHPLC-MSMS method (Stationary phase Kinetex Polar C18 100 × 4.6 mm, 2.6 µm protected with security guard column EVO C18 3 mm ID. The mobile phase was composed of 65 % 5 mmoL/L ammonium formate buffer and 35 % methanol with 0.2 % formic acid with the flow rate 0.6 mL/min). Based on routine monitoring of patient's health status, markers such as C-reactive protein, were monitored, and parameters at admission such as nucleocapsid protein of SARS-CoV-2 – number of viral antigens in the serum (viral N-antigen, NAg) [19, 20] and PCR cycle threshold values (CT, viral copies/mL) were evaluated. NAg was determined by SARS-CoV-2 Antigen quantitative assay kit (Fluorescence Immunochromatography) from Biohit Healthcare (Helsinki, Finland), and CRP was measured using COBAS analyser Roche Diagnostics GmbH (Mannheim, Germany). In addition, some parameters were included in the evaluation, such as body mass index (BMI), age, and clinical frailty scale (CFS) [21].

The disease severity was defined by the need for oxygen therapy, and patients were divided into two groups according to the need for subsequent oxygen therapy.

The prognosis was evaluated by dividing the patients based on survival or death during hospitalization. None of the patients was discharged with the fatal infection (i.e. all patients with terminal COVID-19 treated with palliative intent died in the hospital).

Statistical analysis

Data were processed by NCSS (Kaysville, UT, USA) statistical software for the correlation analysis, nonparametric Mann-Whitney test, and logistic regression. Receiver-operating characteristic (ROC) curves were constructed. The Wald test (Wald Chi-Squared Test) aparametric statistical measure was used to confirm whether a set of independent variables is collectively significant for a model or not.

The Wald test is a statistical test used in logistic regression to determine if a predictor variable is significantly associated with the outcome variable. It calculates a statistic based on the estimated coefficient and its standard error and compares it to a critical value (p=0.05). If the statistic is higher than the critical value, the predictor variable is considered significant.

Canonical correlation analysis, a statistical method for identifying linear relationship between two sets of variables was used [22]. Original datasets coming from lognormal distribution were normalized for analysis by log transformation. This was done to prevent interference by out-layers and heteroscedasticity. In Canonical correlation, there are two sets of variables, x and y. The variables were divided into two groups, i.e. serum biomarkers and urine biomarkers. The goal was to find a linear combination of variables in x and a linear combination of variables in y that have the highest possible correlation between them. These linear combinations urine and blood are called canonical variates (CV) urine1=a1y1 + a2y2 + a3y3 + a4y4, blood1=b1x1 + b2x2 + b3x3 + b4x4.

The coefficients a, and b are called loadings. Loadings are also paired with correlation coefficients (r) between CV and each variable. The first canonical variate (CV1) is found by maximizing the correlation between the linear combination of x variables and the linear combination of y variables. The second canonical variate (CV2) is found by maximizing the correlation between the linear combination of x variables (after removing the influence of the CV1) and the linear combination of y variables (after removing the influence of the CV1). The number of CV is equal to the smaller of the number of variables in x and the number of variables in y. Each canonical variate has an associated canonical correlation coefficient, which measures the strength of the relationship between the two sets of variables.

Results

Initial urinary and serum concentrations of neopterin, kynurenine and tryptophan measured with the first 4 days of hospitalization are presented in Table 2. As expected, the concentrations differed markedly from normal values established in earlier studies [23–26].

Table 1: Patients group characteristics.

		Delta	Omicron	Entire cohort[a]
Gender (M/F)		38/22	22/16	65/43
Age (median), years		69	71.5	72.5
Dead				
During hospitalization		14	9	25
Subsequently to hospitalization within one year after virus detection		3	1	5
CFS (median)		3	4	4
BMI (median)		27.35	28.25	28.25
Oxygen therapy	Yes	42	22	69
(During hospitalization)	No	18	16	39
Corticosteroids	Yes	41	23	69
Before first sample collection	No	19	15	39
Renal insufficiency	Yes	15	9	25
(Initial)	No	45	29	83
Comorbidities				
Hepatic disease		8	2	10
Cancer		10	13	25
Diabetes mellitus		19	19	41
Arterial hypertension		39	27	73
Cardiovascular disease		20	20	43

M, male; F, female; CFS, clinical frailty scale; BMI, body mass index, [a]the entire cohort comprises patients with positive SARS-CoV-2 PCR test with omicron, delta, or variant not defined.

Table 2: Urinary and serum concentrations of target markers in the study group of hospitalized patients.

		Delta		Omicron		Entire cohort[a]	
		n=59		n=39		n=108	
		Median	Range	Median	Range	Median	Range
S neopterin	nmol/L	46.64	8.69–735.14	40.50	9.66–640.04	44.46	7.45[a]–735.14
S kynurenine	μmol/L	4.57	1.74–15.00	4.53	1.82–15.47	4.54	1.68[a]–15.47
S tryptophan	μmol/L	42.62	6.52–63.78	41.89	12.78–76.54	42.26	6.52–76.54
S kynurenine/tryptophan	mmol/mol	105.82	30.23–689.27	108.40	30.49–692.77	106.44	30.23–692.77
U neopterin/creatinine	μmol/mol	953.19	333.25–4,354.58	881.65	257.54–6,333.00	914.93	167.19[a]–6,333.00
U kynurenine/creatinine	mmol/mol	2.69	0.08–43.96	2.34	0.11–14.54	2.57	0.08–43.96
U kynurenine/tryptophan	mmol/mol	556.37	21.81–4,206.21	483.06	38.04–6,690.54	534.32	21.81–6,690.54
U tryptophan/creatinine	mmol/mol	4.91	0.68–45.13	6.07	1.51–16.20	5.63	0.68–45.13
C-reactive protein	mg/L	69.95	1.90–325.40	38.25	3.40–338.40	58.15	1.90–338.40
Viral N-antigen	ng/L	455.81	8.09–12,733.30	41.38	8.92–15,453.00	171.33	8.09–15,453.00

S, serum; U, urine; [a]the entire cohort comprises patients with positive SARS-CoV-2 PCR test with omicron, delta, or variant not defined.

Correlation between urinary and serum biomarkers

The correlation between urinary and serum markers is shown in Table 3. The correlation was determined in the entire cohort of patients and separately in the subgroups of patients infected with delta and omicron virus variants. Urinary levels were always corrected to creatinine levels or evaluated as a ratio product/substrate (kynurenine/tryptophan).

A strong canonical correlation between serum and urine samples was observed ($r=0.87$; $p<0.0001$) that explains 90.3 % of variation in the dataset. Canonical variate from serum samples is most affected by neopterin and kynurenine/tryptophan. Canonical variate from urine samples is mostly affected by neopterin/creatinine ratio (Figure 2). Another canonical correlation is not considered as important, because of explaining only 7.9 % of variance and 1.1 % of variance.

Table 3: Linear correlation between analytes in serum and urine analyzed by Pearson correlation coefficient r, ($p \leq 0.05$).

Parameter	Pearson correlation r	p-Value
S neopterin – U neopterin/creatinine ratio	0.775	<0.0001
S kynurenine/tryptophan ratio – U neopterin/creatinine ratio	0.633	<0.0001
S kynurenine/tryptophan ratio – U kynurenine/tryptophan ratio	0.640	<0.0001
S kynurenine – U kynurenine/tryptophan ratio	0.689	<0.0001
S neopterin – S kynurenine	0.724	<0.0001
S neopterin – S kynurenine/tryptophan ratio	0.799	<0.0001
S kynurenine – S kynurenine/tryptophan ratio	0.836	<0.0001
S tryptophan – S kynurenine/tryptophan ratio	–0.714	<0.0001
U kynurenine/tryptophan ratio – U kynurenine/creatinine ratio	0.809	<0.0001

S, serum; U, urine.

A strong correlation was observed between urinary and serum neopterin concentrations. A correlation was also observed between serum kynurenine/tryptophan ratio and urinary neopterin (r=0.633, p<0.0001), serum kynurenine and urinary kynurenine/tryptophan ratio (r=0.689, p<0.0001). The kynurenine/tryptophan ratio also correlates in urine and serum (r=0.640, p<0.0001). The results also indicated a strong correlation in the serum of neopterin and kynurenine (r=0.724, p<0.0001) and neopterin and kynurenine/tryptophan ratio (r=0.799, p<0.0001) (Figure 3).

Association of the urinary and serum biomarkers with the severity of the disease

The disease severity was defined by the need for oxygen therapy, and patients were divided into two subgroups according to the need for subsequent oxygen therapy.

Serum biomarkers

In the entire cohort, statistically significantly higher levels of all serum biomarkers (neopterin, kynurenine and kynurenine/tryptophan ratio) were observed in the patients with the need for further oxygen therapy (Table 4).

In patients infected with the delta variant same trend of statistically significantly increased serum levels of kynurenine and kynurenine/tryptophan ratio were noted. In patients infected with the omicron variant, the difference did not reach statistical significance.

Urine biomarkers

All investigated urinary biomarkers were significantly increased in patients who subsequently needed oxygen therapy in the entire cohort and patients infected with the delta variant. In the patients infected with the omicron variant, the difference did not reach statistical significance (Table 5).

There were no statistically significant differences in serum neopterin (p=0.4303) and urinary markers (neopterin p=0.3439, kynurenine p=0.4778, kynurenine/tryptophan p=0.3575) with and without oxygen therapy in the patients with corticosteroid therapy before sampling (n=69). Kynurenine and kynurenine/tryptophan ratio still demonstrated statistically significantly higher levels in the group with oxygen therapy (p=0.0286, p=0.0150).

NAg and CRP were also evaluated as biomarkers for prediction of disease severity. NAg levels admission were significantly higher in the patients who needed subsequent oxygen therapy (median 648.99, range 8.92–15,453.00 vs. 33.80 ng/L, 8.92–2,643.00 ng/L; p=0.0019). CRP concentrations were significantly higher in patients who needed oxygen therapy (87.0 mg/L, 3.2–338.4 vs. 27.1, 1.9–128.3 mg/L; p<0.0001).

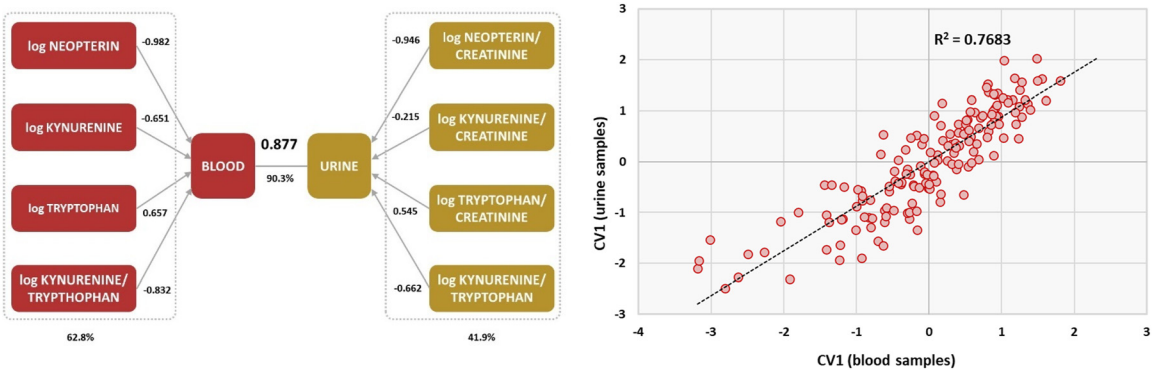

Figure 2: Canonical correlation of studied biomarkers between serum and urine samples LEFT: correlation coefficient r=0.877, p<0.0001 indicates a strong canonical correlation between serum and urine samples explaining 90.3 % of variance from the datasets. The numbers next to rectangulars are individual correlation coefficients of selected analytes and percentage of variance from the datasets RIGHT: determination of the coefficient R2 characterizes the quality of the regression model CV, canonical variate; R2, determination coefficient.

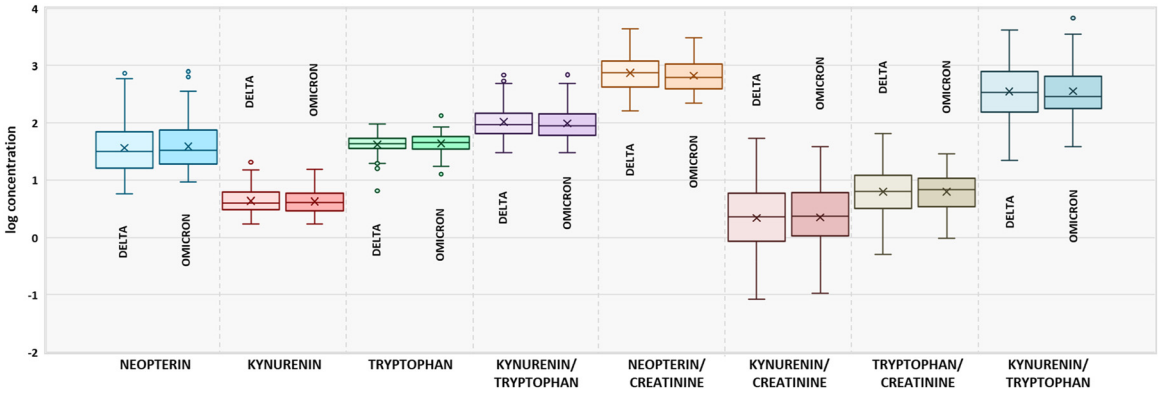

Figure 3: Comparison of biomarker concentrations in patients infected with the omicron and delta virus variants.

Table 4: Differences in selected serum markers at admission according the need of subsequent oxygen therapy (p≤0.05).

		Neopterin, nmol/L		Kynurenine, µmol/L		Tryptophan, µmol/L		Kynurenine/tryptophan ratio, mmol/mol	
Delta	Oxygen	Median (range) 55.21 (8.69–735.14)	p=0.1121	Median (range) 5.19 (1.74–15.00)	**p=0.0034**	Median (range) 40.94 (6.52–58.15)	p=0.2622	Median (range) 125.50 (30.23–689.27)	**p=0.0017**
	No oxygen	Median (range) 35.85 (11.33–151.52)		Median (range) 3.71 (2.20–6.19)		Median (range) 41.38 (32.57–63.78)		Median (range) 76.39 (37.89–169.12)	
Omicron	Oxygen	Median (range) 53.90 (9.66–640.04)	p=0.2198	Median (range) 4.95 (1.82–15.47)	p=0.211488	Media (range) 41.93 (12.78–76.54)	p=0.7117	Median (range) 125.05 (30.49–692.77)	p=0.3067
	No oxygen	Median (range) 32.81 (12.60–128.40)		Median (range) 4.35 (1.88–7.57)		Median (range) 42.79 (23.58–73.41)		Median (range) 98.32 (38.36–218.42)	
Entire cohort[a]	Oxygen	Median (range) 51.39 (8.69–735.14)	**p=0.0202**	Median (range) 5.02 (1.74–15.47)	**p=0.0002**	Median (range) 42.62 (6.52–76.54)	p=0.2616	Median (range) 125.50 (30.23–692.77)	**p=0.0009**
	No oxygen	Median (range) 31.94 (7.45[a]–151.52)		Median (range) 3.65 (1.68[a]–7.57)		Median (range) 41.79 (23.58–73.41)		Median (range) 92.33 (37.58[a]–218.42)	

[a]In the entire cohort are patients with positive SARS-CoV-2 PCR test with omicron, delta, and not defined variant. Bold value means statistically significant result (p≤0.05).

A total of 69 patients started corticosteroids before the first sampling. In this subgroup of patients, only serum kynurenine and kynurenine/tryptophan ratio were significantly increased in patients who needed subsequent oxygen therapy while differences in serum neopterin and all investigated urinary biomarkers did not reach statistical significance (data not shown).

In a small subgroup of patients with renal failure (n=25) no statistical significance could be detected according to the need for subsequent oxygen therapy when analysing the urinary biomarkers, serum kynurenine and kynurenine/tryptophan ratio were significantly increased in patients who needed subsequent oxygen therapy (data not shown). Evaluation of serum markers cut-off values in patients with renal failure could be the aim of the future research.

Urinary and serum biomarkers as the predictors of the disease prognosis

The median hospitalization time in all patients was 14 days, 14 days in survivors (n=83) and 27 days in patients who died (n=25).

Serum biomarkers measured at the start of the hospitalization were significantly higher in patients who died compared to survivors (Table 6) and urinary biomarkers showed the same trend (Table 7).

NAg levels were also statistically significantly higher in patients who died (median 732.18 ng/L, range 8.09–12,733.30 ng/L vs. 91.54 ng/L, 8.92–15,453 ng/L; p=0.0158), but no difference between survivors and patients who died was observed for the cycle viral threshold values-CT (data not

Table 5: Differences in selected urinary biomarkers at admission between patients with future oxygen therapy and without this therapy (p≤0.05).

		Neopterin/creatinine ratio, μmol/mol		Kynurenine/creatinine ratio, mmol/mol		Tryptophan/creatinine ratio, mmol/mol		Kynurenine/tryptophan ratio, mmol/mol	
Delta	Oxygen	Median (range) 1,107.76 (333.25–4,021.33)	**p=0.0138**	Median (range) 3.86 (0.08–43.96)	**p=0.0039**	Median (range) 5.55 (0.68–45.13)	p=0.2233	Median (range) 663.36 (21.81–4,206.21)	**p=0.0133**
	No oxygen	Median (range) 848.26 (403.52–1,201.32)		Median (range) 1.49 (0.22–5.93)		Median (range) 3.64 (1.29–13.17)		Median (range) 339.21 (52.60–1,526.07)	
Omicron	Oxygen	Median (range) 972.55 (257.54–6,333.00)	p=0.2550	Median (range) 2.67 (0.11–14.54)	p=0.6889	Median (range) 6.23(1.51–16.20)	p=0.7562	Median (range) 541.34 (38.04–6,690.54)	p=0.7562
	No oxygen	Median (range) 633.24 (280.57–2,547.45)		Median (range) 2.78 (0.18–12.06)		Median (range) 5.68 (1.75–14.52)		Median (range) 392.89 (104.79–3,529.55)	
Entire cohort[a]	Oxygen	Median (range) 1,013.78 (257.54–6,333.00)	**p=0.0019**	Median (range) 6.26 (0.08–43.96)	**p=0.0036**	Median (range) 6.04 (0.68–45.13)	p=0.1465	Median (range) 605.70 (21.81–6,690.54)	**p=0.0180**
	No oxygen	Median (range) 692.11 (167.19[a]–2,547.45)		Median (range) 1.55 (0.18–12.06)		Median (range) 4.85 (1.29–14.52)		Median (range) 315.15 (52.60–3,529.55)	

[a]The entire cohort comprises patients with positive SARS-CoV-2 PCR test with omicron, delta, and not defined variant. Bold value means statistically significant result (p≤0.05).

Table 6: Differences in selected serum markers at admission between survivors and patients who died (p≤0.05).

		Neopterin, nmol/L		Kynurenine, μmol/L		Tryptophan, μmol/L		Kynurenine/tryptophan ratio, μmol/mmol	
Delta	Survived	Median (range) 37.00 (8.69–735.14)	**p=0.0075**	Median (range) 4.34 (1.74–13.48)	p=0.0814	Median (range) 42.96 (15.91–63.78)	p=0.4164	Median (range) 103.76 (30.23–689.27)	**p=0.0363**
	Died	Median (range) 101.53 (24.38–589.92)		Median (range) 6.33 (2.43–15.00)		Median (range) 39.94 (6.52–54.66)		Median (range) 150.95 (76.18–547.76)	
Omicron	Survived	Median (range) 33.68 (9.66–120.68)	**p=0.0012**	Median (range) 4.31 (1.82–8.37)	**p=0.0046**	Median (range) 45.56 (23.58–76.54)	**p=0.0074**	Median (range) 97.06 (30.49–239.15)	**p=0.0012**
	Died	Median (range) 128.40 (21.50–640.04)		Median (range) 7.57 (3.26–15.47)		Median (range) 33.00 (12.78–48.60)		Median (range) 293.30 (78.06–692.77)	
Entire cohort[a]	Survived	Median (range) 34.00 (7.45[a]–735.14)	**p=<0.0001**	Median (range) 4.05 (1.68[a]–13.48)	**p=0.0002**	Median (range) 44.29 (15.91–76.54)	**p=0.0156**	Median (range) 97.88 (30.23–689.27)	**p=<0.0001**
	Died	Median (range) 107.23 (15.36[a]–640.04)		Median (range) 6.46 (2.43–15.47)		Median (range) 36.37 (6.52–54.66)		Median (range) 190.55 (76.18–692.77)	

[a]In the entire cohort are patients with positive SARS-CoV-2 PCR test with omicron, delta, and not defined variant. Bold value means statistically significant result (p≤0.05).

Table 7: Differences in selected urinary markers at admission between survivors and patients who died (p≤0.05).

		Neopterin/creatinine ratio, nmol/mmol		Kynurenine/creatinine ratio, µmol/mmol		Tryptophan/creatinine ratio, µmol/mmol		Kynurenine/tryptophan ratio, µmol/mmol	
Delta	Survived	Median (range) 987.22 (333.25–2,871.32)	p=0.2709	Median (range) 1.92 (0.08–14.54)	p=0.1728	Median (range) 4.94 (0.83–29.22)	p=0.7201	Median (range) 507.49/(21.81–2095.36)	p=0.1468
	Died	Median (range) 1,058.74 (504.14–4,354.58)		Median (range) 4.74 (0.13–43.96)		Median (range) 4.08 (0.68–45.13)		Median (range) 719.85 (93.30–4,206.21)	
Omicron	Survived	Median (range) 655.54 (257.54–2,547.45)	**p=0.0024**	Median (range) 1.68 (0.11–14.54)	p=0.1979	Median (range) 6.39 (1.75–16.20)	p=0.0860	Median (range) 278.43 (38.04–6,690.54)	**p=0.0078**
	Died	Median (range) 1,541.47 (432.73–6,333.00)		Median (range) 5.46 (1.00–9.20)		Median (range) 3.45 (1.51–8.73)		Median (range) 1,187.21 (267.71–3,529.55)	
Entire cohort[a]	Survived	Median (range) 840.16 (167.19[a]–2,871.32)	**p=0.0018**	Median (range) 1.78 (0.08–14.54)	**p=0.0158**	Median (range) 5.71 (0.83–29.22)	p=0.2076	Median (range) 370.15 (21.81–6,690.54)	**p=0.0010**
	Died	Median (range) 1,257.49 (306.93[a]–6,333.00)		Median (range) 5.41 (0.13–43.96)		Median (range) 4.05 (0.68–45.13)		Median (range) 787.89 (93.30–4,206.21)	

[a]In the entire cohort are patients with positive SARS-CoV-2 PCR test with omicron, delta, and not defined variant. Bold value means statistically significant result (p≤0.05).

shown). In CT values wasn't this trend observed (0.1639, died patients: median 19.59 viral copies/mL, range 14.50–37.90 viral copies/mL, survivors: median 21.69 viral copies/mL, range 11.79–37.50 viral copies/mL).

We also calculated ROC curves with defined sensitivity and specificity prediction concerning the subsequent need for oxygen therapy and survival. ROC curves were evaluated for individual biomarkers and the biomarker combined with other factors such as age, CFS at admission, body mass index, and routinely measured parameters such as CRP or NAg.

ROC curves and survival

The highest area under the curve (AUC) values were for urinary neopterin (0.7069), urinary kynurenine/tryptophan ratio (0.7180), and serum kynurenine/tryptophan ratio (0.7899).

Among the combinations with other parameters, the highest AUC was observed for the combination of serum kynurenine and BMI (0.8390), serum kynurenine, age and BMI (0.8264), serum kynurenine and age (0.7983), and urinary neopterin, BMI, and age (0.7958). AUC values for NAg and CRP were 0.6804 and 0.7376, respectively.

The survival cut-off level evaluation

To evaluate cut-off levels for survival it was necessary to classify all surviving patients with 100 % sensitivity to make sure that none of the survivors would be predicted to die (with zero false negative results) because of the different treatment strategy between these groups (category of the care).

For the urinary neopterin as a single parameter the cut-off value (equal and higher means died) was 2,881 µmol/mol creatinine with 100 % sensitivity (Wald 11.36), for urinary kynurenine the cut-off was 15 mmol/mol creatinine (Wald 3.47), for serum kynurenine the cut-off value was 13.6 µmol/L (Wald 12.68), and for serum tryptophan the cut-off (equal and higher means survived) was 15.8 µmol/L (Wald 6.29).

Regarding the combination of the biomarkers with other parameters, the cut-off value for the index obtained by the equation A calculated from the parameters including urinary neopterin, NAg, and age was set as 0.54 (with higher values predicting death) resulting in an AUC of 0.8439 (Figure 4). In this combination, the impact of individual parameters, expressed as Wald value, was demonstrated for age (Wald 9.31), urinary neopterin (Wald 8.38), and NAg (Wald 1.87). Other combinations using serum biomarkers that may be used with 100 % specificity comprise age (Wald 8.54),

serum tryptophan (Wald 3.11), and NAg (Wald 1.77) with cut-off value of 0.60 and AUC of 0.8047 (calculated using equation B). Another combination in serum with 100 % sensitivity was NAg (Wald 2.07), tryptophan (Wald 1.47) and kynurenine (Wald 11.69) with cut-off value of 0.80 and AUC 0.8453 (equation not shown).

The subsequent need for oxygen therapy cut-off values evaluation

Similar to the survival, parameters were tested individually and in combinations. Sensitivity and specificity values were set to obtain a minimum of false positive and negative results. For individual parameters predicting the need for future oxygen therapy, the highest AUC values were observed for serum kynurenine (0.7224), serum CRP (0.7881), and urinary neopterin (0.6807).

For the combination of investigational biomarkers in serum with routine parameters the best results were obtained for an index combining serum neopterin (Wald 2.71), serum tryptophan (Wald 8.74), serum kynurenine/tryptophan ratio (Wald 5.04), BMI (Wald 1.07), NAg (Wald 1.93) and CRP (Wald 9.16), with a cut-off value 0.52 AUC of 0.8859 (Figure 5). Among 85 patients were 16 falsely classified (77 % sensitivity and 83 % specificity [equation C]).

The best combination of investigational biomarkers in urine included tryptophan (Wald 6.32), CFS (Wald 1.75), BMI (Wald 0.58), NAg (Wald 5.41) and CRP (Wald 6.83) with the cut-off value set as 0.61 (higher values mean subsequent need for oxygen therapy), the AUC of 0.8777 and the specificity 81 % and sensitivity 85 % (equation D, Figure 4).

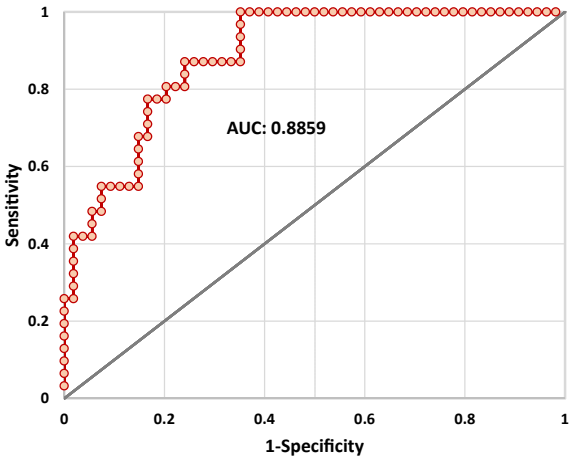

Figure 5: ROC curve: combination serum neopterin, tryptophan, kynurenine/tryptophan ratio, NAg, and CRP. AUC-area under curve.

Discussion

Present data support the utilization of neopterin, kynurenine, and tryptophan as biomarkers in the management of patients with COVID-19. Increased neopterin, kynurenine and kynurenine/tryptophan ratio were associated with the severity of the disease. Among different scales and approaches that may be used to assess disease severity in patients with COVID-19 the need for subsequent oxygen therapy was selected because of the obvious significance in planning the therapy during the pandemic [27]. For the first time, we evaluated concentrations of urinary and serum neopterin, kynurenine, and tryptophan separately in

A

$$P_{death} = \frac{1}{1 + e^{-(-10.619130 + 0.101287 * age + 0.000117 * NAg + 0.001383 * urinary\ neopterin)}}$$

B

$$P_{death} = \frac{1}{1 + e^{-(-6.234610 + 0.000106 * NAg - 0.048077 * serum\ tryptophan + 0.090842 * \mathrm{age})}}$$

C

$$P_{oxygen\ therapy} = \frac{1}{1 + e^{-(-10.375329 - 0.017213 * \mathrm{serum\ neopterin} + 0.127464 * \mathrm{serum\ tryptophan} + 0.025849 * \mathrm{serum\ kynurenine/tryptophan} + 0.057416 * \mathrm{BMI} + 0.000706 * \mathrm{NAg} + 0.025468 * \mathrm{CRP})}}$$

D

$$P_{oxygen\ therapy} = \frac{1}{1 + e^{-(-4.399462 + 0.205631 * \mathrm{urinary\ tryptophan} + 0.208745 * \mathrm{CFS} + 0.037874 * \mathrm{BMI} + 0.001216 * \mathrm{NAg} + 0.017658 * \mathrm{CRP})}}$$

Figure 4: Equations for cut-off values calculation. All equations were calculated by logistic regression, a statistical method used for binary classification problems, where the goal is to predict the probability of an event occurring based on one or more input variables. Logistic regression uses a logistic function to map the linear combination of the predictor variables to a value between 0 and 1, which can be interpreted as the probability of a binary event occurring.

patients infected with the delta and omicron variants [10] and noted a correlation between serum neopterin and kynurenine/tryptophan ratio suggestive of an association between COVID-19 severity with tryptophan catabolism. The present data are in agreement with this study. We looked into the possibility of using neopterin, kynurenine and tryptophan independently and in combination with commonly routinely measured biomarkers such as CRP, CT, and NAg and clinical parameters such as age, CFS, and BMI. For all determinations of the present set of inflammatory biomarkers, highly selective and sensitive techniques of high performance liquid chromatography were used in combination with mass spectrometry or fluorescence and photodiode array detection. In contrast to prior studies that were carried out using immunochemical methods, which, as is generally known, are associated with many drawbacks, such as crossreactivity and the possibility to determine only a single analyte, while the price is comparable with chromatographic techniques, the present investigations were carried out using HLPC methods. In addition, the investigational biomarkers assessed in the present study have been previously evaluated only in serum. One of the principal aims of this study was to address the possibility of using urine as an alternative sample matrix. The utilization of urine has an obvious advantage because of non-invasive sample collection, less burden for the patient and more simple processing. A strong virus variant independent correlation was observed between urinary and serum biomarker concentrations. We observed a correlation of serum and urinary neopterin, serum kynurenine/tryptophan ratio and urinary neopterin, serum kynurenine and urinary ratio kynurenine/tryptophan and urinary ratio kynurenine/tryptophan and serum ratio kynurenine/tryptophan in the whole study group of the patients with COVID-19. In the serum, we also confirmed the results of Geisler et al. [28], who published a correlation between neopterin and kynurenine in different viral infections. Thus, both serum and urine can be used as sample matrix.

Marked differences in serum and urinary neopterin, kynurenine and kynurenine/tryptophan ratio were observed based on the subsequent oxygen therapy in the whole cohort and the delta variant, but the differences did not reach formal statistical significance in patients infected with the omicron variant, probably because of limited sample size.

Present data also demonstrate that neopterin, kynurenine and kynurenine/tryptophan ratio may be used to predict short term mortality in COVID-19. These biomarkers were markedly higher in patients who died compared to surviving patients in the whole cohort as well as when the patients infected in the delta or omicron variant were examined separately. The prediction of short-term mortality has important implications for clinical practice as patients with an extremely high risk of death may be offered new experimental therapy or, in the situation of capacity limitations, priority for intensive care resources could be directed to patients with a better chance of survival.

As was reported by Zheng et al. [6], corticosteroid therapy suppressed serum neopterin production in SARS-CoV-2 patients. Our results are consistent with these findings. We observed no statistically significant differences in serum neopterin and urinary markers with and without oxygen therapy in the patients with corticosteroid therapy before sampling. But kynurenine and kynurenine/tryptophan ratio still showed statistically significantly higher levels in the group with oxygen therapy. Therefore, the solution is urine sampling before corticosteroid therapy starts or serum kynurenine and kynurenine/tryptophan ratio usage.

Neopterin and tryptophan concentrations along with other parameters like age or nucleocapsid antigen level may be useful components in the calculation of a multiple parameter index assessing the need of oxygen therapy or risk of death. The selection of an optimal set of biomarkers should be investigated in future studies. These studies should also try to integrate the biochemical biomarkers like neopterin or kynurenine with direct measures of the host immune response. The current approach for the assessment of response to SARS-CoV-2 relies mostly on the measurement of antibodies directed to the virus, but the measurement of cellular immune response may be more relevant [27, 29]. Future studies should investigate the correlation between neopterin and kynurenine production and tests directly assessing cellular immune response in SARS-CoV-2 infection.

The present retrospective study has several potential limitations. The number of subjects in subgroups of patients was sometimes limited, and an absence of statistical significance in these subgroups may reflect lack of statistical power rather than an absence of an effect. The number of patients also did not allow to split the subjects into a learning and validation cohort, and these results should be validated in future studies. Some of the patients started corticosteroid therapy before the sample collection, and this may have obscured the inflammatory response. Using the need for oxygen therapy as an outcome measure may be prone to subjective bias of treating physician as well as resource availability. However, none of the patients who did not receive oxygen died, and in the setting of a pandemic necessitating rational use of resources the use of such clinical decision may provide a higher degree of reliable

information compared to even more bias-prone measures affected by subjective assessment such as different severity scores. Circulating SARS-CoV-2 variants are evolving rapidly, and emerging variants have different biology and clinical presentation. The host response may also be different. However, similar results were observed in the present study for two SARS-CoV-2 variants. Although the cut-off values for the need for oxygen therapy or short-term prognosis may differ with newly emerging variants, present data indicate that the determination of neopterin, kynurenine and tryptophan may be useful not only in COVID-19, but also in other severe respiratory infections [6, 30, 31].

In conclusion, present data demonstrate that neopterin, kynurenine and kynurenine/tryptophan ratio in the serum or in the urine represent promising biomarkers in the management of COVID-19 that may help to guide important therapeutic decisions.

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