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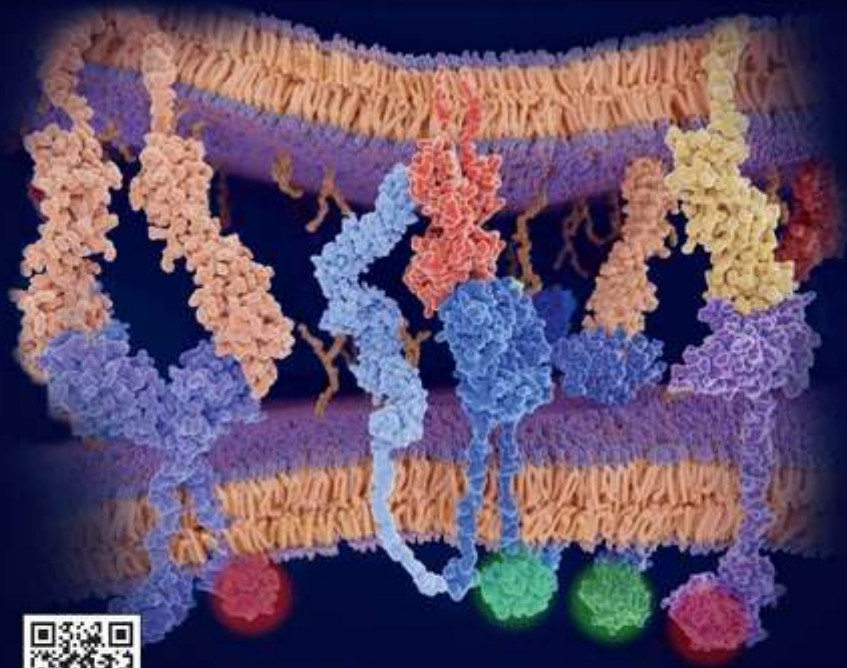
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Цитокиновые паттерны летальных гипервоспалительных состояний, индуцированных вторичным гемофагоцитарным синдромом, бактериальным сепсисом и COVID-19

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Резюме

Введение. При тяжелом течении коронавирусной инфекции 2019 (COVID-19) образование легочных инфильтратов сопровождается синдромом «цитокинового шторма» (ЦШ). Помимо COVID-19, причины развития ЦШ включают вторичный гемофагоцитарный синдром (ВГФС) и септический шок (СШ).

Цель данного исследования – сравнение иммунологических профилей пациентов с неблагоприятными исходами COVID-19, ВГФС и СШ.

Материал и методы. Сывороточные концентрации ИЛ-1β, ИЛ-2, ИЛ-6, ИЛ-8, ИЛ-10, ИЛ-17A, ИЛ-18, ИФН-γ, ФНОα, прокальцитонина, неоптерина, ферритина с определением процента гликозилирования (% ГФ) определяли проспективно у 37 пациентов с COVID-19, поступивших в стационар и скончавшихся в 2020 г.; ретроспективно у 39 пациентов с ВГФС и у 47 пациентов с СШ, поступивших в стационар и скончавшихся за 2018–2019 гг. Группы сравнения также включали образцы сыворотки крови 194 пациентов с благоприятными исходами COVID-19 и 20 здоровых доноров, собранные в течение 2020 г. Концентрации цитокинов, прокальцитонина и неоптерина измеряли с помощью иммуноферментного анализа; уровень ферритина определяли турбидиметрическим методом. Для расчета % ГФ была использована модификация метода M. Wogwood и соавт.

Результаты. У пациентов с летальными исходами COVID-19 медианы концентраций ИЛ-6, ИЛ-8, ИЛ-10, ИЛ-18 и прокальцитонина были достоверно выше, чем при благоприятных исходах заболевания. В то же время концентрации ИЛ-8, ИЛ-18, ИФН-γ, ФНОα и ферритина были достоверно ниже при летальном COVID-19 по сравнению с ВГФС и СШ. Концентрации ИЛ-6 и прокальцитонина у пациентов с COVID-19 были достоверно выше, чем при ВГФС, но не отличались от соответствующих показателей в группе СШ. Медиана числа лейкоцитов была наиболее высокой в группе летального COVID-19 ($p < 0,05$).

Заключение. Каждый вариант летальной гипервоспалительной реакции сопровождался характерными особенностями цитокинового профиля: высокие концентрации ИЛ-6 в сочетании с низкими концентрациями ИФН-γ, ФНОα при COVID-19; высокие концентрации ИЛ-6, ИЛ-8 и низкие ИЛ-17A, ИЛ-2 при СШ; высокие концентрации ИЛ-18 и ИФН-γ, а также ферритина, и низкие концентрации ИЛ-6, прокальцитонина, низкие показатели % ГФ при ВГФС.

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Ключевые слова: COVID-19; гипервоспаление; «цитокиновый шторм»; вторичный гемофагоцитарный синдром; септический шок

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Cytokine patterns of fatal hyperinflammatory conditions, caused by secondary hemophagocytic lymphohistiocytosis, bacterial sepsis and COVID-19

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Abstract

Introduction. In severe cases of coronavirus disease 2019 (COVID-19) pulmonary infiltration is accompanied by cytokine storm syndrome (CSS) development. Besides COVID-19, CSS can be triggered by the range of pathologies, which include hemophagocytic lymphohistiocytosis (sHLH) and septic shock (SS).

The aim of this study was to compare immunological profiles in fatal cases of COVID-19, sHLH and SS.

Material and methods. Serum levels of IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-17A, IL-18, IFN- γ , TNF- α , procalcitonin, neopterin, ferritin with percent of glycosylated fraction (% GF) were measured in 37 COVID-19 fatal cases, collected during 2020 year prospectively; and in 39 sHLH and 47 SS fatal cases, collected within 2018–2019 years retrospectively. Comparison groups also included 194 non-fatal COVID-19 cases and 20 healthy donors, collected during 2020 year. Cytokine concentrations, procalcitonin and neopterin were measured by enzyme-linked immunosorbent assay; the ferritin level was determined by the turbidimetry method. The percent of glycosylated ferritin fraction (% GF) was calculated by the modified method of M. Worwood et al.

Results. Deceased patients with COVID-19 had higher IL-6, IL-8, IL-10, IL-18, procalcitonin median levels compared to the survived. Meanwhile IL-8, IL-18, IFN- γ , TNF α and ferritin concentrations were significantly lower in deceased COVID-19 patients compared to sHLH and SS. The levels of IL-6 and procalcitonin in fatal COVID-19 were comparable to SS, but significantly higher than in sHLH. Leucocytes were higher in COVID-19 compared to both SS and sHLH.

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Conclusion. Each fatal condition was accompanied by specific features of the cytokine profile: high IL-6 combined with low IFN- γ , TNF α in COVID-19; high IL-8, IL-6 with low IL-17A, IL-2 in SS; high IL-18, ferritin, IFN- γ with low IL-6, procalcitonin, % GF in sHLH.

Keywords: COVID-19; hyperinflammation; «cytokine storm syndrome»; secondary hemophagocytic lymphohistiocytosis; septic shock

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Introduction

Inflammation is a universal protective mechanism that activates in response to a pathogen invasion. In some pathologies the immune system inability to eliminate pathogen can lead to its overstimulation and cytokine storm syndrome (CSS) development [1]. This severe condition is characterized by overwhelming systemic inflammation and hemodynamic instability, leading to a multiple organ dysfunction syndrome

(MODS) and fatal outcomes in many cases. Common manifestations include sustained fever, splenomegaly, hepatomegaly with liver dysfunction, lymphadenopathy, coagulopathy, cytopenia, skin rash, and variable neurologic symptoms [2]. The documented conditions causing CSS are hemophagocytic lymphohistiocytosis (HLH), septic shock (SS), toxin-mediated shock syndrome (e.g., staph toxic shock), some viral infections and graft versus host disease [3].

Table 1. Demographic parameters of patients with fatal COVID-19, SS and sHLH outcomes

Parameter	sHLH (n = 39)	SS (n = 47)	COVID-19 (n = 37)
Etiology	<ul style="list-style-type: none"> · Hematological malignancy: 28/39 (71.8 %), · Intracellular-pathogen induced infections (<i>Leishmania</i> spp., <i>HIV</i>, <i>Epstein-Barr virus</i>): 6/39 (15.4 %), · Undefined aetiology: 5/39 (12.8 %) 	<ul style="list-style-type: none"> · <i>Klebsiella pneumoniae</i>: 10/47 (21.3 %), · <i>Acinetobacter baumannii</i>: 5/47 (10.6 %), · <i>Staphylococcus epidermidis</i>: 6/47 (12.8 %) · <i>Staphylococcus hominis</i>: 3/47 (6.4 %), · <i>Pseudomonas aeruginosa</i>: 3/47 (6.4 %), · <i>Streptococci</i> spp.: 3/47 (6.4 %), · <i>Enterobacter cloacae</i>: 1/47 (2.1 %), · <i>Enterococcus faecium</i>: 1/47 (2.1 %), · <i>Esherichia coli</i>: 1/47 (2.1 %) 	SARS-CoV-2: 37/37 (100.0 %)
Age, years	58.0 (31.0–73.0)	65.0 (43.7–77.2)	69.0 (58.5–82.0)
Gender, male/female	19/20	30/17	24/13
Cause of death	<ul style="list-style-type: none"> · MODS: 24/39 (61.6 %), · Hematological tumor progression: 13/39 (33.3 %), · Stroke: 2/39 (5.13 %) 	MODS: 47/47 (100.0 %)	<ul style="list-style-type: none"> · ARDS: 27/37 (73.0 %), · MODS 8/37 (21.6 %), · Hyperglycemic coma 1/37 (2.7%), · AMI 2/37 (5.4 %)
Pathogenetic treatment	<ul style="list-style-type: none"> · Conventional chemotherapy · Etoposide · Intravenous immunoglobulin · Cyclosporine A · Glucocorticosteroids 	Antibacterial treatment (according to local policy)	<ul style="list-style-type: none"> · Anti-cytokine drugs (ruxolitinib or tocilizumab) · Immunosuppressive

sHLH – secondary Hemophagocytic Lymphohistiocytosis; SS – Septic Shock (caused by bacteria); COVID-19 – coronavirus disease 2019; SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2; ARDS – Acute Respiratory Distress Syndrome; AMI – Acute Myocardial Infarction; MODS – Multiple Organ Dysfunction Syndrome.

Table 2. Clinical and biochemical parameters of patients with COVID-19, SS and sHLH

Parameter	Reference values	sHLH (fatal outcome)	SS (fatal outcome)	COVID-19 (fatal outcome)	COVID-19 (favorable outcome)	sHLH/SS	sHLH / COVID-19	SS/ COVID-19	COVID-19 fatal/ favorable outcome
% GF, %	> 78.3	19.9 (4.4–34.2)	40.0 (30.5–54.5)	40.5 (22.7–57.7)	60.5 (46.3–76.3)	***	**	ns	***
Ferritin total, µg /L	Men: 20.0–250.0. Women: 10.0–120.0	10741.0 (7488.0–18547.0)	1801.0 (892.3–3507.0)	1243.0 (758.0–2113.0)	605.5 (334.5–895.8)	****	****	ns	****
Procalcitonin, pg/ml	< 0.25	0.04 (0.02–1.55)	2.13 (0.65–3.58)	0.58 (0.17–1.08)	0.14 (0.06–0.20)	**	*	ns	***
Neopterin, nmol/L	< 10.0	70.3 (36.7–138.5)	110.6 (57.4–146.0)	56.6 (31.77–110.1)	30.4 (24.7–37.8)	ns	ns	*	***
C-reactive protein, mg/L	1.0–5.0	224.0 (98.4–310.0)	147.0 (57.9–200.0)	144.5 (50.2–244.0)	60.0 (16.9–136.4)	ns	ns	ns	***
WBC, x10 ⁹ /L	4.00–8.80	3.40 (1.13–7.03)	6.97 (3.18–17.98)	12.89 (9.76–16.23)	7.23 (4.98–10.00)	ns	****	ns	****
Neutrophils, x10 ⁹ /L	2.20–4.80	3.35 (2.0–5.0)	5.64 (3.22–14.73)	11.64 (7.45–14.11)	5.63 (3.26–8.78)	ns	****	*	****
Lymphocytes, x10 ⁹ /L	1.20–2.50	0.70 (0.44–1.16)	1.02 (0.43–1.66)	0.70 (0.42–1.40)	1.0 (0.8–1.6)	ns	ns	ns	**

Here and in tables 3, 4: the reference value for % GF was calculated in previous study [11]. sHLH – secondary Hemophagocytic Lymphohistiocytosis; SS – Septic Shock (caused by bacteria); COVID-19 – coronavirus disease 2019; WBC – White Blood Cells; % GF – the percent of Glycosylated Ferritin fraction. The significance level *p* for each pair is represented as «ns» at a value > 0.05 (not significant); * – *p* < 0.05; ** – *p* < 0.01; *** – *p* < 0.001; **** – *p* < 0.0001.

In March 2020 the World Health Organization announced a pandemic of new coronavirus disease 2019 (COVID-19) [4], which in most severe cases was characterized by pulmonary infiltration, accompanied by CSS [5]. Some common features were found in CSS caused by COVID-19 and secondary HLH (sHLH) due to the severity of their clinical course, frequent lung involvement [6], and similar immunological profile including elevated IL-1β, IL-2, IL-8, IL-10, IL-17, TNF-α, IFN-γ et al. [7]. Furthermore, both COVID-19 and HLH also had common immunological and clinical characteristics with SS, caused by bacterial infection [8, 9]. Although an elevation of multiple inflammatory parameters is essential for all listed conditions, some of them could be combined and serve as patterns, reflecting immunopathological characteristics of underlying disease.

Hyperferritinemia is an additional sign of COVID-19, SS, and especially HLH when it can exceed the upper reference limit by more than a hundred times [10]. Sooner the percent of glycosylated ferritin fraction (% GF) was reported as more specific marker to differentiate sHLH and SS compared to a total ferritin level, with a threshold value of 30.4 [11]. However there is still no data whether % GF decreases in COVID-19 and whether inflammatory markers may be combined into specific pattern to distinguish CSS in COVID-19 from other hyperinflammatory conditions.

The aim of this study was to compare immunological profiles in fatal cases of COVID-19, sHLH and SS.

Material and methods

Patients. This study was approved by the biomedical ethics committee of the I.P. Pavlov First Saint Petersburg State Medical University (protocol No. 233). Serum samples, demographic and clinical data were collected from 37 fatal cases of COVID-19 prospectively during 2020 year. The median number of days from admission to the intensive care unit and taking blood until the end point was 6.5 days, with 7 day case-fatality rate (CFR) of 50.0 % and 28-day CFR of 93.3 %. The diagnosis of COVID-19 was confirmed in all patients with viral pneumonia by PCR tests. Concomitant pathologies in COVID-19 patients included hypertensive heart disease (67.57 %, 25/37), coronary heart disease (59.46 %, 22/37), chronic heart failure (29.73 %, 11/37), diabetes mellitus (18.92 %, 7/37), chronic obstructive pulmonary disease (10.81 %, 4/37), malignancies (21.62 %, 8/37) and chronic kidney disease (18.92 %, 7/37). To estimate the difference between inflammatory status in fatal and non-fatal COVID-19 cases, serum samples of 194 survived COVID-19 patients were also collected.

Serum samples and clinical data of 39 sHLH and 47 SS fatal cases that had been collected within 2018–2019 years were analyzed retrospectively. The median number of days from admission and taking blood until the end point was 6.0 days in sHLH and 1.0 day in SS. 7- and 28-day CFRs consisted 50.0 and 75.0 % in sHLH, and 95.4 and 100.0 % in

Table 3. Cytokine concentrations in patients with COVID-19, SS and sHLH

Parameter	Healthy donors	sHLH (fatal outcome)	SS (fatal outcome)	COVID-19 (fatal outcome)	COVID-19 (favorable outcome)	sHLH / SS	sHLH / COVID-19	SS/ COVID-19	COVID-19 fatal/ favorable outcome
IL-1 β , pg/ml	0.20 (0.10–0.87)	0.13 (0.1–1.47)	0.36 (0.11–19.95)	0.88 (0.15–1.67)	0.20 (0.01–0.88)	ns	ns	ns	ns
IL-2, pg/ml	0.15 (0.10–0.20)	1.46 (0.32–43.07)	0.01 (0.01–0.14)	0.07 (0.01–0.18)	0.09 (0.05–0.12)	*	ns	ns	ns
IL-6, pg/ml	0.63 (0.25–1.37)	24.23 (7.91–122.90)	99.80 (16.94–512.3)	75.27 (24.30–166.1)	17.94 (8.75–44.74)	**	**	ns	****
IL-8, pg/ml	2.90 (2.21–3.98)	141.3 (74.31–343.5)	498.1 (80.47–643.2)	23.78 (14.86–32.28)	11.70 (6.50–30.57)	ns	****	****	*
IL-10, pg/ml	1.43 (0.89–1.79)	64.70 (17.90–236.8)	42.33 (15.87–500.0)	20.00 (11.16–29.80)	7.69 (4.46–11.0)	ns	*	*	****
IL-17A, pg/ml	4.85 (0.19–21.57)	51.32 (6.89–101.50)	8.11 (0.21–30.53)	31.95 (30.29–33.66)	30.84 (29.74–33.00)	*	ns	ns	ns
IL-18, pg/ml	81.40 (67.06–133.80)	2690.0 (2042.0–2741.0)	1575.0 (942.4–2368.0)	783.9 (537.4–1117.0)	445.8 (287.2–657.9)	*	****	****	****
IFN- γ , pg/ml	0.20 (0.10–0.52)	35.24 (21.00–55.62)	19.35 (11.02–36.61)	4.55 (0.21–10.19)	0.26 (0.10–2.76)	*	****	*	ns
TNF- α , pg/ml	0.71 (0.30–1.04)	36.90 (22.13–60.73)	19.33 (9.58–37.62)	3.77 (2.17–6.11)	3.50 (2.89–4.58)	ns	**	*	ns

sHLH – secondary Hemophagocytic Lymphohistiocytosis; SS – Septic Shock (caused by bacteria); COVID-19 – coronavirus disease 2019.

SS respectively. The sHLH diagnosis was established with the use of HLH-2004 criteria [12] and H-Score [13]. The median number of points was 5.0 (4.0–6.0) by HLH-2004 criteria and 227 (178.5–256.0) by H-Score. The development of sHLH was triggered by Non-Hodgkin's Lymphoma (41.0 %, 16/39), Hodgkin's disease (5.1 %, 2/39), multiple myeloma (15.4 %, 6/39), acute leukemia (10.3 %, 4/39), infections (15.4 %, 6/39), and in 12.8 % cases (5/39) etiology wasn't defined.

The diagnosis of sepsis was established according to ACCP/SCCM criteria [14] and confirmed based on the positive blood culture result in 57.45 % (27/47) cases. The median value on the Sequential Organ Failure Assessment (SOFA) Score in SS patients consisted 12.0 points. Primary foci of inflammation were identified in all patients with SS and were presented by severe pneumonia (61.7 %, 29/47), peritonitis (25.5 %, 12/47), sinusitis (6.4 %, 3/47), skin infections (4.3 %, 2/47) and meningoencephalitis (2.1 %, 1/52).

Laboratory research. Serum samples from 20 healthy donors were taken in order to estimate normal cytokine levels. Following inflammatory parameters were measured by the enzyme-linked immunosorbent assay (ELISA) with the use of commercial kits made by OOO «Vector-Best» (Russia) for

IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-18 and IFN- γ ; OOO «Cytokine» (Russia) for IL-17A; RayBiotech (USA) for procalcitonin and IBL International GmbH (Germany) for neopterin. Total ferritin content was determined by turbidimetric method with the use of AU480 chemistry analyzer (Beckman Coulter, USA). The percent of glycosylated ferritin fraction (% GF) was calculated by the M. Worwood's method modification [15].

Statistical analyses were performed with GraphPad Prism 9 (GraphPad Software, LLC, version 9.1.0 for Windows 64-bit). The quantitative results are presented as median with an interquartile range Me (Q1–Q3). Nonparametric data were compared by Kruskal-Wallis one-way analysis of variance. Dunn's pairwise multiple comparison posttest was used to compare each patient group. Correlations between the parameters were evaluated using Spearman's rank correlation test. To assess the diagnostic significance of the deviating parameters ROC-analysis (Receiver Operating Characteristic) was used. Diagrams were prepared by the online editor Visual Paradigm Online (<https://online.visual-paradigm.com/>).

Results

Patients with sHLH were younger ($p < 0.05$) compared to other groups and suffered from hematological malignan-

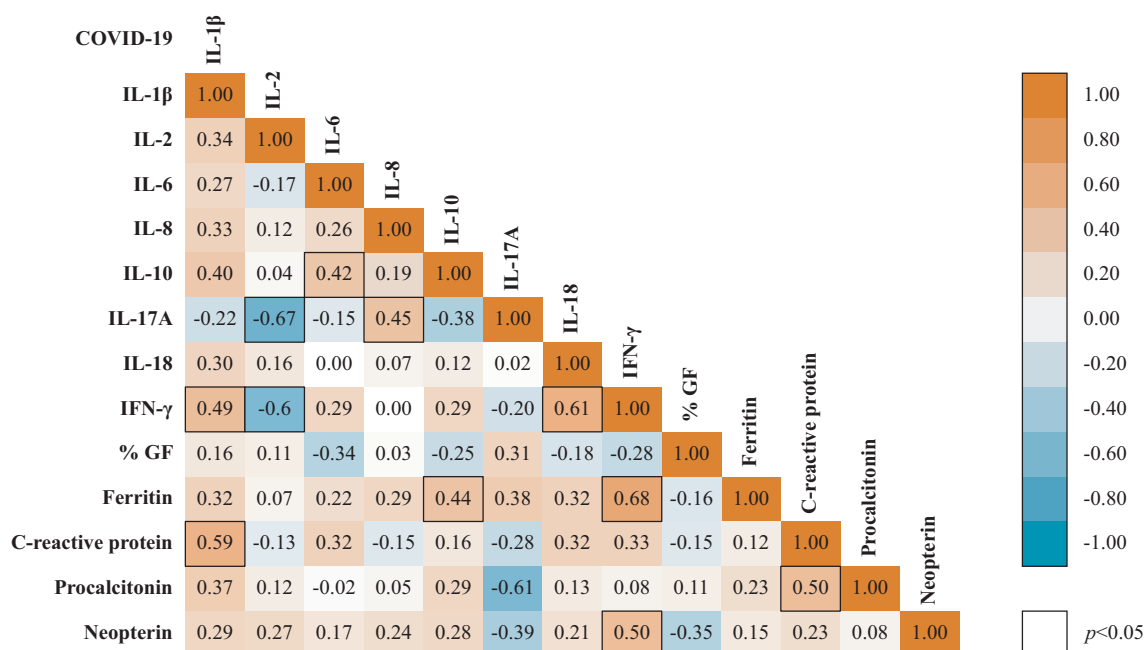


Fig. 1. Correlation matrix showing the Spearman's correlation coefficient between inflammatory biomarkers pairs in COVID-19 fatal cases *COVID-19 – coronavirus disease 2019; % GF – the percent of glycosylated ferritin fraction.*

cies in 28/39 (71.8%) cases, which played a role of a trigger in sHLH. Demographic data from fatal cases of COVID-19, SS and sHLH are presented in a table 1.

Clinical and laboratory parameters of patients with different COVID-19 outcomes, fatal SS and sHLH are presented in a table 2.

Survived patients with COVID-19 had significantly lower procalcitonin, ferritin, C-reactive protein, neopterin,

WBC and higher % GF median values, compared to the deceased. Highly elevated ferritin levels with % GF reduction were found in all fatal hyperinflammatory conditions, though in sHLH these changes were much more pronounced compared to other groups. Meanwhile, seven deceased patients with COVID-19 had % GF levels below the threshold value for sHLH diagnosis, but with moderately elevated

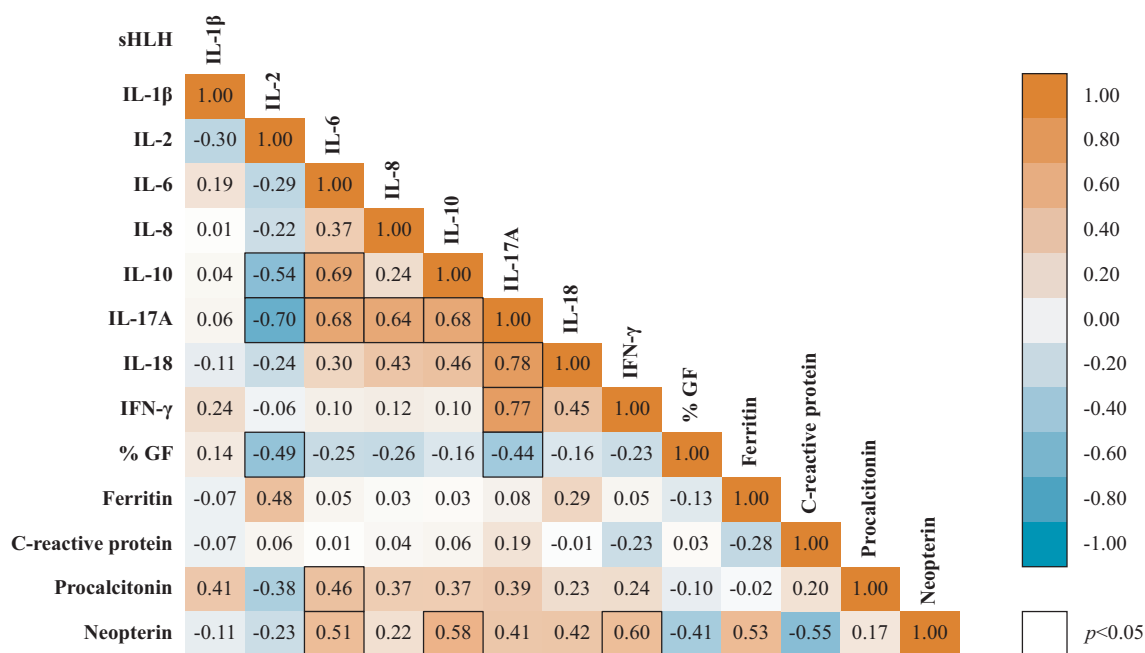


Fig. 2. Correlation matrix showing the Spearman's correlation coefficient between inflammatory biomarkers pairs in sHLH group *sHLH – secondary Hemophagocytic Lymphohistiocytosis; % GF – the percent of glycosylated ferritin fraction.*

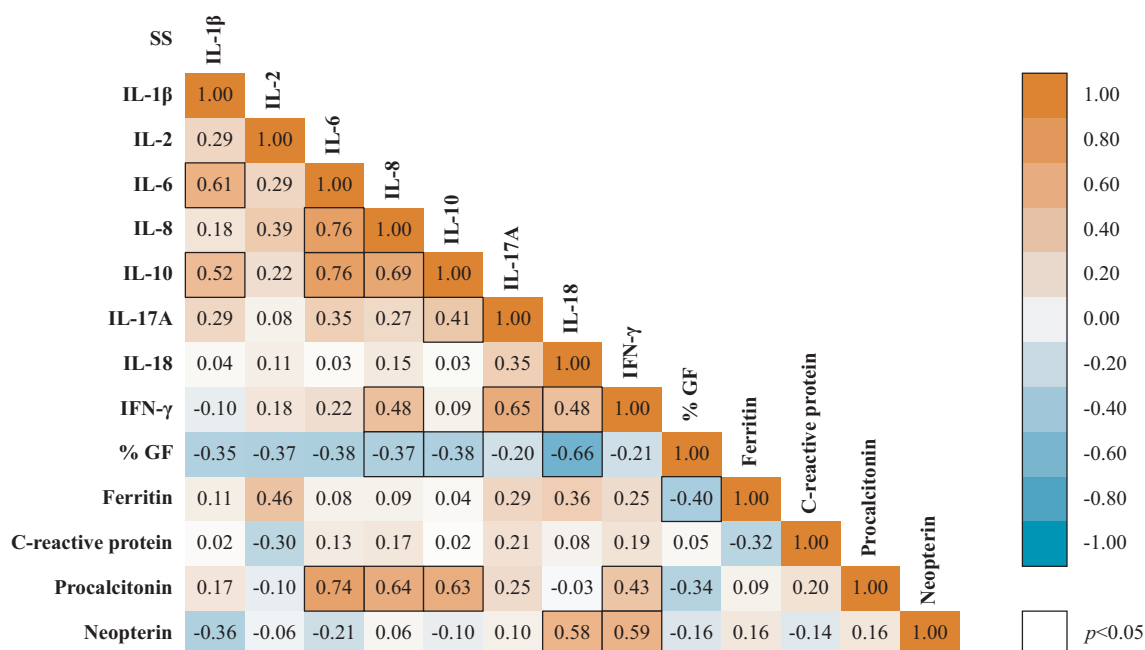


Fig. 3. Correlation matrix showing the Spearman's correlation coefficient between inflammatory biomarkers pairs in SS group SS – Septic Shock (caused by bacteria); % GF – the percent of glycosylated ferritin fraction.

total ferritin concentrations. The WBC count was the highest in deceased COVID-19 patients and normal in sHLH.

Serum cytokine levels in patients with different COVID-19 outcomes, fatal SS and sHLH are presented in a table 3.

According to the data presented in the table 3 hypercytokinemia was less pronounced in COVID-19 as com-

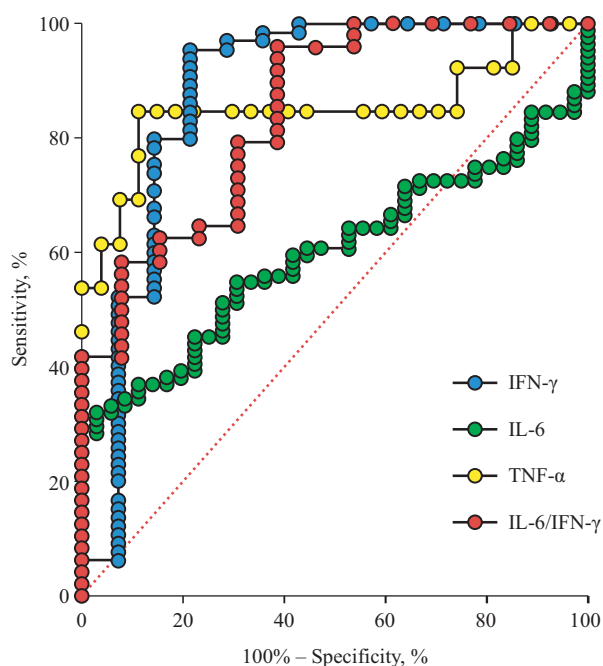


Fig. 4. ROC-curves of parameters with the highest (IL-6) and lowest (IFN- γ , TNF- α) medians, as well as IL-6/IFN- γ ratios for COVID-19 group with unfavorable outcomes

pared to other hyperinflammatory conditions. The median levels of IL-8, IL-10, IL-18, IFN- γ and TNF- α were significantly lower in fatal COVID-19 than in sHLH and SS groups. At the same time median concentrations of IL-6, IL-8, IL-10 and IL-18 were significantly higher in deceased COVID-19 compared to the survived.

The results of correlation analysis of cytokines and inflammatory biomarkers in COVID-19, sHLH and SS fatal cases are presented in fig. 1–3.

The correlation analysis of COVID-19 group parameters revealed the pairs with strong positive correlation: IL-1 β /IFN- γ , IL-1 β /C-reactive protein, IL-8/IL-17A, IFN- γ /ferritin, IFN- γ /neopterin, C-reactive protein/procalcitonin; and strong negative correlation: IL-2/IL-17A, IL-2/IFN- γ , IL-17A/procalcitonin.

The correlation matrix of sHLH group revealed the cluster containing IL-6, IL-17A, IL-10 and IL-18 that positively correlated to each other (the only exception was IL-18/IL-6). The cluster of negative correlations contained the pairs of IL-2 and % GF (IL-2/ % GF, IL-2/IL-10, IL-2/IL-17A, % GF-IL-17A). In sHLH group IL-6 concentrations correlated with both procalcitonin ($r = 0.46$, $p = 0.040$) and neopterin ($r = 0.51$, $p = 0.037$). Meanwhile IL-10 and IFN- γ correlated with neopterin ($r = 0.51$, $p = 0.037$ for IL-6, $r = 0.58$, $p = 0.018$ for IL-10), but did not correlate with procalcitonin. The levels of IFN- γ correlated positively with a number of points on the HLH-2004 scale ($r = 0.48$, $p = 0.030$) and neopterin ($r = 0.60$, $p = 0.019$) in sHLH patients.

The correlation analysis of SS group also showed the cluster of positively correlating parameters at the top of the matrix (IL-6/IL-8, IL-6/IL-10, IL-6/IL-1 β , IL-8/IL-10, and IL-1 β /IL-10). The «blue line» of % GF pairs contained significantly negative correlations with IL-8 ($r = -0.37$, $p =$

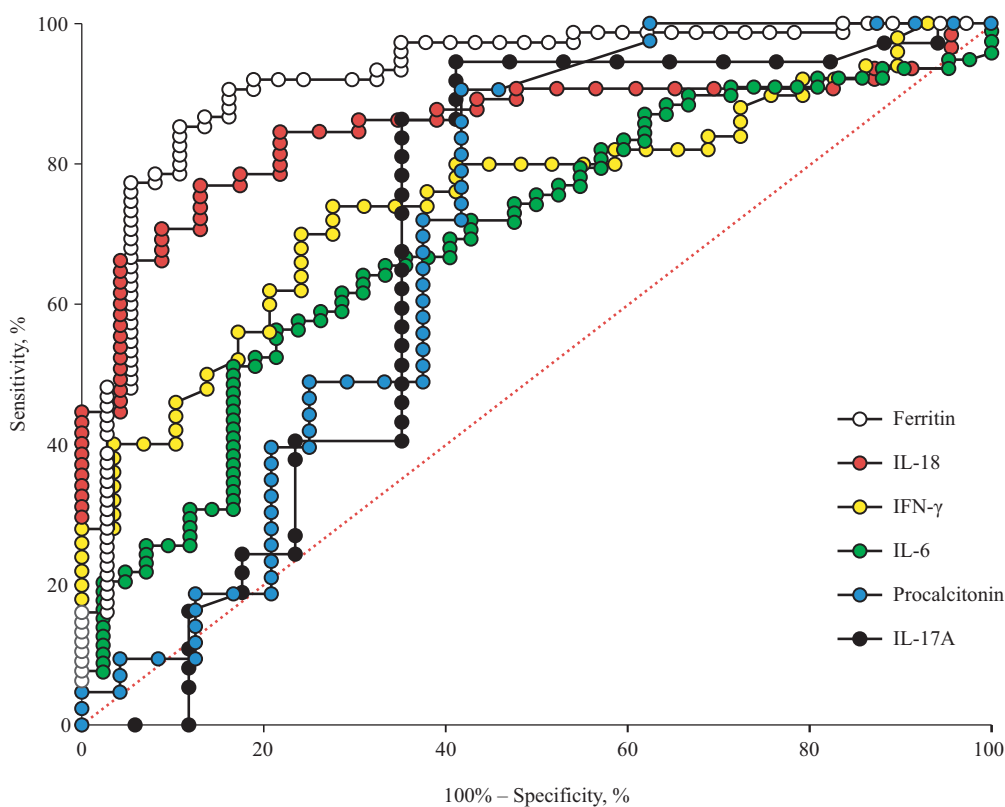


Fig. 5. ROC-curves of parameters with the highest (ferritin, IL-18, IFN- γ , IL-17A) and lowest (IL-6, procalcitonin) medians for sHLH group with unfavorable outcomes

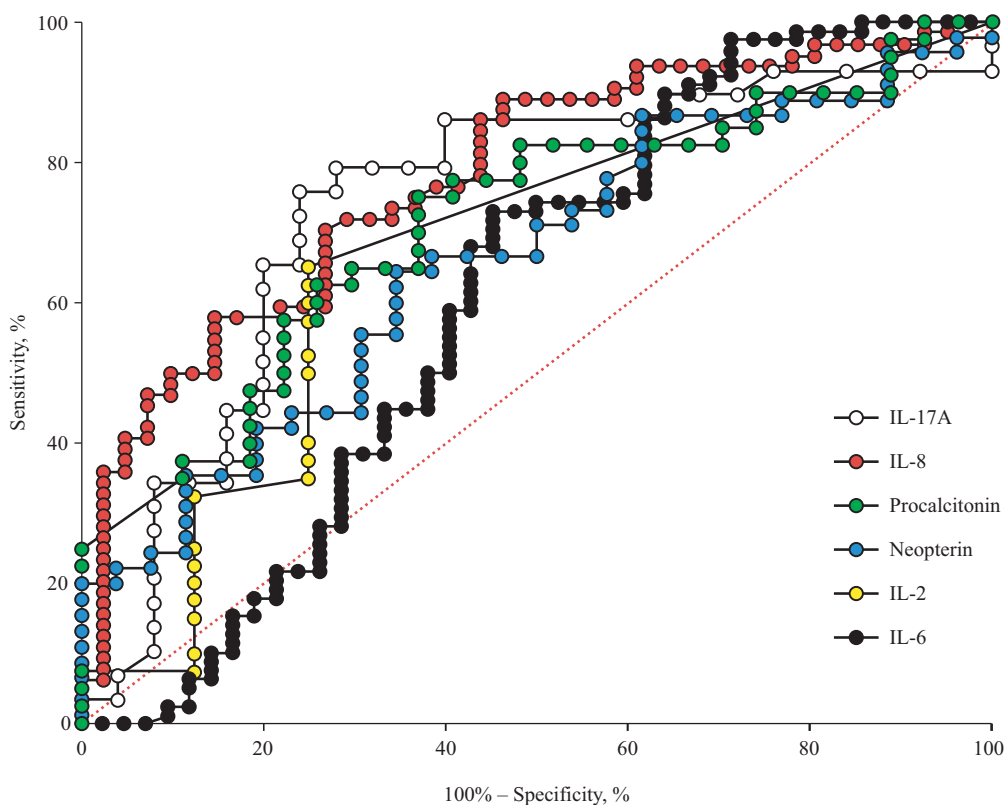


Fig. 6. ROC-curves of parameters with the highest (IL-8, procalcitonin, neopterin, IL-6) and lowest (IL-17A, IL-2) medians for SS group with unfavorable outcomes

Table 4. The diagnostic characteristics of the most increased and decreased parameters of patients with unfavorable COVID-19, sHLH and SS outcomes

Parameter	Area under the curve	Cut-off value	Sensitivity, %	Specificity, %	PLR
<i>Group of patients with COVID-19</i>					
IFN- γ , pg/ml	0.8780	< 25.04	52.31	92.86	7.32
TNF- α , pg/ml	0.8519	< 26.52	61.54	96.30	16.62
IL-6, pg/ml	0.5982	> 11.91	32.14	97.22	11.57
IL-6/IFN- γ	0.8446	> 3.49	58.33	92.31	7.583
<i>Group of patients with sHLH</i>					
Ferritin total, $\mu\text{g/L}$	0.9196	> 3204.0	77.33	94.59	14.31
IL-18, pg/ml	0.8528	> 1425.0	66.15	95.65	15.22
IFN- γ , pg/ml	0.7510	> 11.43	40.00	96.55	11.60
IL-6, pg/ml	0.6943	< 413.7	20.51	97.62	8.61
Procalcitonin, pg/ml	0.7025	< 0.057	90.70	58.33	2.18
IL-17A, pg/ml	0.6852	> 35.33	86.49	64.71	2.45
<i>Group of patients with SS</i>					
IL-17A, pg/ml	0.7407	< 48.87	34.48	92.0	4.31
IL-8, pg/ml	0.7795	> 31.96	40.63	95.12	8.33
Procalcitonin, pg/ml	0.7148	> 0.057	37.50	88.89	3.37
Neopterin, nmol/L	0.6594	> 30.99	22.22	96.15	5.78
IL-2, pg/ml	0.6703	< 0.025	65.00	75.00	2.60
IL-6, pg/ml	0.6036	> 87.81	73.08	54.76	1.61

PLR – positive likelihood ratio.

0.047), IL-10 ($r = -0.38$, $p = 0.048$), IL-18 ($r = -0.66$, $p = 0.004$) and ferritin ($r = -0.40$, $p = 0.020$). Similar to sHLH matrix, IL-17A showed strong positive correlation with IFN- γ ($r = 0.65$, $p = 0.001$). Unlike sHLH matrix, IL-2 did not show any significant correlations in SS group. Positively correlating pairs were created by procalcitonin and neopterin with cytokines (procalcitonin/IL-6, procalcitonin/IL-8, procalcitonin/IL-10, neopterin/IL-18, neopterin/IFN- γ), but they did not correlate to each other. Besides laboratory parameters, IL-6 showed positive correlation with the number of points on the SOFA scale ($r = 0.56$, $p = 0.008$).

To assess the diagnostic characteristics of the most increased and decreased parameters of patients with unfavorable COVID-19, sHLH and SS outcomes the ROC-analysis was performed. The graphical results of ROC-analysis are shown in fig. 4–6; the quantitative data are presented in a table 4.

According to the ROC-analysis low serum levels of IFN- γ and TNF α are specific for unfavorable COVID-19 course. Moreover, this patient group was characterized by higher IL-6/IFN- γ compared to patients with sHLH and SS. Ferritin, IL-18, IFN- γ and a decrease in IL-6 had the highest specificity for diagnosing sHLH-associated CSS, and the area under the curve was the largest for ferritin and IL-18. In the SS group, the area under the curve was largest for IL-8 (highest median in the SS group) and IL-17A (lowest median in the SS group). The low specificity of the increase in IL-6 is due to the high concentrations of IL-6 in the group of patients with unfavorable outcomes of COVID-19.

Discussion

Hypercytokinemia is an essential component of systemic inflammation of any etiology, though the proportions of certain parameters may vary depending on the pathogen

type, underlying disease and patient's individual features. Severe bacterial sepsis and sHLH are life-threatening conditions with similar clinical and laboratory manifestations and an excessive inflammatory response that leads to multiple organ failure and if not promptly treated, to fatal outcome. The development of CSS in sepsis is associated with dysfunctional immune response provoked by circulating infectious agents and their toxins in the bloodstream [16]. sHLH is manifested by the development of an autoinflammatory syndrome with uncontrolled macrophage activation in response to the trigger in addition to defective cytotoxic immunity, which leads to the inability of CD8⁺-T-lymphocytes or NK cells to eliminate intracellular pathogen [17]. Cytotoxic immunity disorders have also been identified in severe COVID-19 [18, 19], when the progression of infection and the accumulation of damage-associated molecular patterns (DAMPs) are accompanied by lymphopenia, neutrophilia, and suppression of IFN- γ -mediated immunity [20, 21]. In the current study the unfavorable COVID-19 course was also accompanied by neutrophilia and lymphopenia, while IFN- γ was lower than in SS and sHLH, but wasn't different compared to recovered patients with COVID-19. The most probable causes of lymphopenia and low IFN- γ concentrations are the ability of SARS-CoV-2 to suppress both interferon types I and II expression in monocytes and epithelial cells [22], as well as functional exhaustion of NK cells and CD8⁺-T-lymphocytes [23].

The distributions of inflammatory markers in deceased patients with COVID-19, sHLH and SS and in different COVID-19 outcomes (fatal vs non-fatal) are presented in fig. 7, 8.

According to the data presented in the fig. 7 each hyperinflammatory condition had distinct increased and de-

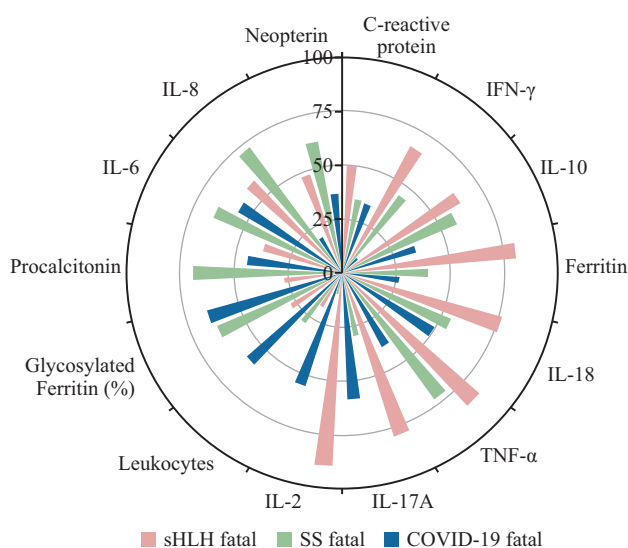


Fig. 7. Immunological parameters of patients with fatal COVID-19, sHLH and SS outcomes

Median values of laboratory parameters are transformed into percentile values of these parameters in total group of patients with fatal conditions.

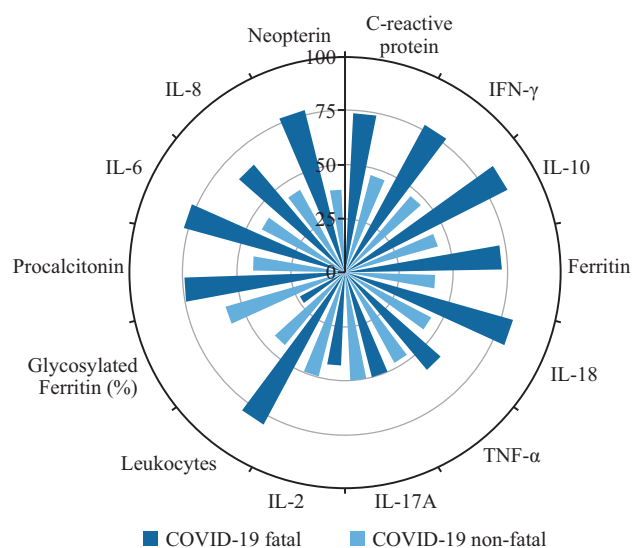


Fig. 8. Immunological parameters of patients with fatal and non-fatal COVID-19 outcomes

Median values of laboratory parameters are transformed into percentile values of these parameters in total COVID-19 group.

COVID-19 – coronavirus disease 2019; sHLH – secondary Hemophagocytic Lymphohistiocytosis; SS – Septic Shock (caused by bacteria).

creased parameters. Moreover, despite deceased COVID-19 patients had significantly higher IL-6, IL-8, IL-10, IL-18, procalcitonin, C-reactive protein and neopterin levels compared to survived, some of these parameters were significantly lower compared to SS and sHLH (see fig. 7, 8).

Deceased patients with COVID-19 and SS had comparable IL-6, ferritin, and % GF values. The median levels of IL-6, IL-17A, WBC were high and of IFN- γ , TNF- α , IL-8, IL-2 were low in patients with fatal COVID-19 outcomes. According to ROC-analysis, the sensitivity of IFN- γ , TNF- α , as well as IL-6/IFN- γ ratio was 52.31, 61.54 and 58.33 % respectively, with more than 90 % specificity for differential diagnosis with SS and sHLH (see table 4). Previously Bert N.L. et al. reported an imbalanced response in symptomatic COVID-19 patients with elevated IL-6, TNF- α , IL-1 β and IL-10, but decreased IL-2 and IFN- γ production, whereas asymptomatic patients had proportionally high IL-2 and IFN- γ levels [24]. Nevertheless, both decreased [25] and increased [26] IFN- γ levels in severe COVID-19 compared to its mild form were reported.

Patients with SS had the highest IL-6, IL-8 and procalcitonin levels compared to other study groups, though IL-1 β and IL-17A were not elevated. According to ROC analysis the presented parameters did not have sufficiently high sensitivity and specificity for differential diagnosis with COVID-19 and SS. According to microbiological data, all causative agents of SS were extracellular microorganisms in the current study, however some of them: *Pseudomonas aeruginosa* [27] and *Klebsiella pneumoniae* [28] can survive within macrophages. Recently Slaats J. et al. have proposed IL-1/IL-6/C-reactive protein pattern for the immune response to extracellular pathogens [29], and in the current

study only IL-6 was significantly elevated in patients with SS. However, the absence of increase of IL-1 β could be explained by its extremely short half life less than 20 min [30].

Patients with sHLH had the highest medians of IL-18 and ferritin, which are the markers of intracellular pathogen-induced inflammation according to the Slaats J. et al. hypothesis [29]. In addition, according to ROC analysis their sensitivity, specificity, area under the curve and PLR were the highest for differential diagnosis with other hyperinflammatory conditions (see table 4). Hyperinflammation in sHLH is triggered by either intracellular infectious agents (viral, bacterial or protozoan) or malignancies [31], both of them are known to activate IFN- γ mediated cell immunity [32]. Moreover the highest levels of IFN- γ , TNF- α , IL-2, IL-17A and IL-10 were found in this group. Since IL-18 is inducer of IFN- γ synthesis by Th1 cells and some other cells, the combined increase of these two cytokines indicates an activation of cytotoxic immunity, a priority mechanism against intracellular pathogens [33]. Median values of % GF, IL-6 and procalcitonin were low in patients with sHLH-associated CSS. At the same time sHLH patients demonstrated the highest C-reactive protein concentrations, though this protein was proposed as a part of extracellular pathogen-induced profile [29]. Conversely, neopterin was significantly higher in SS compared to COVID-19; despite earlier it was proposed to differentiate viral and bacterial infections. However, other studies didn't reveal the difference of neopterin in bacterial and viral infections [34], though its prognostic role remains undeniable [35]. This purine nucleotide is regarded as a marker of cellular immune response produced by activated macrophages and dendritic cells after stimulation with IFN- γ [36]. In the current study neopterin

correlated positively with IFN- γ in all patient groups. The median level of procalcitonin was the highest in SS and correlated with IL-6, IL-8 and IL-10; the lowest in sHLH and correlated with IL-6, and intermediate in COVID-19 without significant correlations. The production of procalcitonin by C cells of a thyroid is known to increase under IL-1 β , IL-6, TNF- α stimulation and its serum level is used for monitoring sepsis and, since recently, COVID-19 [37].

The comparative analysis showed that IL-17A median level was the highest in sHLH, the lowest in SS and intermediate in COVID-19 fatal cases. This proinflammatory cytokine mediates neutrophil recruitment in different types of inflammation and is produced by Th17 cells, differentiating in presence of IL-6 and TGF- β [38]. Elevated IL-17A levels in sHLH and COVID-19 patients may reflect its controversial role in immune response against intracellular pathogens. According to the latest data, IL-17 promotes intracellular microorganisms persistence and downregulate T cell cytotoxicity [39], however, it was shown to reduce intracellular pathogen load in a range of infections [40]. The proportions of IL-6 and IL-17A were different in all groups: low IL-6 and high IL-17A in sHLH, high IL-6 and high IL-17A in fatal COVID-19, and high IL-6 and low IL-17A in SS. The effects of IL-17 are known to include endothelial cell activation and stimulation of IL-8 synthesis, a potent chemokine for neutrophils [41]. Despite low IL-17A level, the group with SS demonstrated very high IL-8 concentrations, which can possibly be explained by the presence of bacteria in a bloodstream, stimulating epithelial cells to

activate and produce IL-8 without participation of immune cells [42]. At the same time in some severe pathologies (e.g. COPD) low serum IL-8 levels were reported, that can be explained by its local production and consumption by activated lung endothelial cells [43].

The median level of IL-10 was elevated in all patients with fatal hyperinflammatory conditions, though it was much less pronounced in fatal COVID-19 group compared to sHLH and S.S. Meanwhile, IL-10 concentrations were significantly higher in fatal COVID-19 ($p < 0.0001$) compared to patients with favorable outcomes, and correlated with IL-6. Similar results were reported by Han H. et al. [44]. Elevated IL-10 levels were shown to predict unfavorable outcome in both COVID-19 [45] and sHLH [46]. It was suggested that in COVID-19-associated CSS IL-10 functions not as anti-inflammatory but as proinflammatory cytokine that stimulates an excessive production of virus-induced response mediators [45]. Also IL-10 is presumed to contribute to the resolution of inflammation and a favorable outcome of the disease in infections caused by extracellular bacteria, but hinders elimination and suppresses immune response to intracellular pathogens [47].

Conclusion

Each fatal condition was accompanied by specific features of the cytokine profile: high IL-6 combined with low IFN- γ , TNF- α in COVID-19; high IL-8, IL-6 with low IL-17A, IL-2 in SS; high IL-18, ferritin, IFN- γ with low IL-6, procalcitonin, % GF in sHLH.

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