SAŽETAK

Kod bolesnika s COVID-19 istovremeno su prisutni upala i hipoksija kao signali sa suprotnim učinkom na ekspresiju hepcidina. Istraživanje se temeljilo na hipotezi da je u skupini hipoksičnih bolesnika s COVID-19 koncentracija hepcidina u krvi snižena i da je razina parametara statusa željeza promijenjena u odnosu na skupinu normoksičnih bolesnika. Glavni cilj istraživanja bio je ispitati postoji li razlika u koncentraciji hepcidina i čimbenika koji reguliraju koncentraciju hepcidina kod normoksičnih i hipoksičnih bolesnika s COVID-19 te u skupini zdravih ispitanika. Također, željelo se ispitati postoji li povezanost koncentracije hepcidina i parametara statusa željeza, eritropoeze, hipoksije i sustavne upale u ispitivanim skupinama. Primijenjeni su omjeri koji su uključivali hepcidin i molekule koje su povezane s hepcidinom (hepcidin/željezo, feritin/hepcidin, hepcidin/interleukin-6 (IL-6), hepcidin/C-reaktivni protein (CRP), hepcidin/eritropoetin (EPO)) kako bi se istodobno pratio njihov suodnos i međusoban utjecaj, ali i omjeri kojima se povezuju i stavljaju u suodnos različiti upalni parametri (CRP/IL-6, omjeri CRP-a i IL-6 s leukocitnim podskupinama, omjeri neutrofila i limfocita (NLR), neutrofila i monocita (NMR) te monocita i limfocita (MLR)) kako bi se pokušalo što bolje razlikovati skupine bolesnika s COVID-19 koje imaju različit obrazac kliničke prezentacije i ishode bolesti.

U istraživanje je uključeno 96 bolesnika s COVID-19 i 47 zdravih ispitanika podudarnih po dobi i spolu. Uzorkovanje je provedeno po primitku na Hitni infektološki prijem te su bolesnici s COVID-19 podijeljeni u skupinu normoksičnih i hipoksičnih bolesnika na temelju zasićenje hemoglobina kisikom (SpO_2), a daljnjim praćenjem tijeka bolesti podijeljeni su u skupine prema težini bolesti.

Najvažniji rezultati ovog istraživanja ukazuju da su plazmatske koncentracije hepcidina, feritina, EPO-a, CRP-a i IL-6 te omjeri hepcidin/željezo i feritin/hepcidin bili značajno viši, dok su omjeri hepcidin/IL-6 i hepcidin/CRP bili značajno niži u hipoksičnih u odnosu na normoksične bolesnike te u bolesnika s teškim ili kritičnim oblikom COVID-19 u odnosu na one s blagim i umjerenim oblikom bolesti. Nađena je dobra povezanost hepcidina s CRP-om i IL-6 kod normoksičnih bolesnika, dok je u skupini hipoksičnih bolesnika povezanost s CRP-om bila slaba, a s IL-6 odsutna. Vrijednosti omjera CRP/neutrofilni granulociti, CRP/limfociti, CRP/monociti, NLR i NMR na prijemu bile su najviše kod bolesnika koji su imali teški i kritični tijek bolesti. Omjeri IL-6/neutrofilni granulociti, IL-6/limfociti, IL-6/monociti bili su statistički značajno viši u skupini ispitanika koja je imala kritični tijek bolesti u odnosu na ostale skupne bolesnika. Multiparametarski model za predviđanje razvoja kritičnog oblika COVID-19 koji je uključivao EPO i feritin/hepcidin ispravno je klasificirao 88 % slučajeva.

Prisutnost izraženije sustavne upale u skupini hipoksičnih bolesnika vjerojatno je uzrok više koncentracije hepcidina u odnosu na normoksične bolesnike s COVID-19. Uz to, razlike u vrijednostima pojedinih omjera upalnih biljega među skupinama bolesnika s COVID-19 ukazuju da bi ovi omjeri mogli biti korisni u predviđanju težine bolesti.
Ključne riječi: COVID-19, hepcidin, željezo, feritin, eritropoetin, interleukin 6, hipoksija, upala

EXTENDED SUMMARY

Background: The liver hormone hepcidin is the master regulator of iron metabolism. Hepcidin controls intestinal iron absorption and release of iron from storage compartments. Hepcidin production is regulated on a transcriptional level. Inflammation, high iron stores, and transferrin saturation stimulate hepcidin expression, while anaemia/hypoxia, low iron stores and erythropoietic activity downregulate hepcidin expression. In Coronavirus disease-19 (COVID-19), different factors can affect iron homeostasis. Disturbances of iron metabolism are reported in these patients and are found to be predictors of disease severity and outcome. Iron homeostasis during infection is very important since both pathogens and host organisms need iron. During inflammation, inflammatory cytokines, namely interleukin 6 (IL-6), stimulate hepcidin expression. Hepcidin binds iron exporter ferroportin, causing its internalisation. This leads to iron retention in storage compartments and iron deprivation from pathogenic microorganisms. On the other hand, tissue hypoxia stimulates the expression of erythropoietin (EPO). EPO stimulates erythropoiesis and indirectly leads to inhibition of hepcidin expression. In some COVID-19 patients, inflammation and hypoxia are present simultaneously as signals with opposing effects on hepcidin expression. Studies have shown that hypoxia can overcome the effect of inflammation on hepcidin expression.

Aim: We hypothesised that hypoxia, when present in COVID-19 patients, can overcome the stimulating effect of inflammation on hepcidin expression and can cause decreased concentration of hepcidin in the peripheral circulation of hypoxic patients compared to COVID-19 patients with normal oxygen saturation. The main goal of the present study was to explore the difference in concentration of hepcidin and parameters that regulate hepcidin levels in groups of non-anaemic normoxic and hypoxic COVID-19 patients admitted to the emergency unit before the introduction of therapeutic interventions. Also, we wanted to explore the relationship of hepcidin concentration with parameters that influence hepcidin production: parameters of systemic inflammation (C-reactive protein (CRP), IL-6), the parameter of hypoxia (oxygen saturation (SpO_2)), parameters of erythropoiesis (reticulocyte number (RTC) and EPO concentration) and parameters of iron status (iron (Fe), unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), transferrin saturation (TSAT), ferritin, soluble transferrin receptors (sTfR) and reticulocyte haemoglobin equivalent (RET-He)). Additionally, in order to identify representative surrogates that could indicate dysregulation of iron homeostasis in different patient groups, we applied ratios of hepcidin concentration with parameters of iron metabolism (hepcidin/iron, ferritin/hepcidin), inflammation (hepcidin/CRP, hepcidin/IL-6), and erythropoiesis (EPO). Furthermore, ratios that interconnect and correlate

different inflammatory parameters (CRP/IL-6, CRP/neutrophil leukocytes, CRP/lymphocytes, CRP/monocytes, IL-6/neutrophil granulocytes, IL-6/lymphocytes, IL-6/monocytes, neutrophil to lymphocyte ratio (NLR), neutrophil to monocyte ratio (NMR) and monocyte to lymphocyte (MLR)) were also used in order to distinguish subgroups of COVID-19 patients with different clinical presentations and disease outcomes.

Materials and methods: The study included 96 COVID-19 patients admitted to the emergency unit of the Department of Infectious Diseases and 47 healthy volunteers as a control group. SARS-CoV-2 positivity was confirmed by reverse transcription polymerase chain reaction (RT-PCR) from a nasopharyngeal swab. The inclusion criteria for both groups was age between 20 and 75 years. Strict exclusion criteria were applied to eliminate conditions and diseases that could affect hepcidin concentration and the concentration of other studied parameters. The control group met the same inclusion and exclusion criteria as the patient group except for SARS-CoV-2 positivity. Patients were categorised based on oxygen saturation (SpO_2) measured on admission as hypoxic ($SpO_2 < 94$ %) and normoxic ($SpO_2 \geq 94$ %). Forty-seven hypoxic COVID-19 patients and 49 normoxic patients were matched by sex and age. Also, the healthy control group was matched by sex and age to COVID-19 patients. Additionally, COVID-19 patients were categorised based on prospective follow-up into groups according to the World Health Organization guidelines as patients with mild and moderate, severe and critical forms of the disease.

Demographic, clinical, and anamnestic data were collected upon patients' admission to the Department of Infectious Diseases emergency unit. SpO_2, complete blood count, and routine biochemical tests were analysed. Information on the course of the disease was prospectively monitored by reviewing the medical documentation in the hospital information system. Blood samples for the control group were collected in the Medical Laboratory Diagnostics Division. Haematological parameters (complete blood count (CBC), RTC, IRF, and RET-He) were analysed using the haematology analyser Sysmex XN-1000 (Sysmex Corporation, Kobe, Japan). Plasma concentration of creatinine, iron, UIBC, ferritin, CRP and IL-6 was analysed by standard laboratory methods on analyser Roche Cobas 6000 (Roche Diagnostics GmbH, Manheim, Germany). sTfR concentration was measured nephelometrically on a BN ProSpec analyser (Siemens Healthcare Diagnostics, Marburg, Germany). Commercially available ELISA tests were used for the measurement of hepcidin and EPO concentrations: Hepcidin 25 (bioactive) HS ELISA (DRG Diagnostics GmbH, Marburg, Germany) and Quantikine® IVD® Human Erythropoietin ELISA (R&D Systems Inc., Minneapolis, MN, USA). SpO_2 was measured by an oximetric method on an ABL90 analyser FLEX PLUS (Radiometer,

Copenhagen, Denmark). eGFR was calculated according to the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation. TIBC and TSAT were calculated by the following equations: TIBC = iron (μmol/L) + UIBC (μmol/L); TSAT (%) = iron (μmol/L)/TIBC (μmol/L) × 100. Ratios of hepcidin with parameters of iron metabolism (ferritin/hepcidin, hepcidin/iron), inflammation (hepcidin/CRP, hepcidin/IL-6) and erythropoiesis (hepcidin/EPO) were calculated. Also, different ratios of inflammatory parameters were calculated: CRP/IL-6, CRP/neutrophil leukocytes, CRP/lymphocytes, CRP/monocytes, IL-6/neutrophil granulocytes, IL-6/lymphocytes, IL-6/monocytes, NLR, NMR and MNR.

Data analysis was performed using MedCalc statistical software version 20.013 (MedCalc Software, Ostend, Belgium).

Results: Hepcidin concentration was significantly higher in COVID-19 patients compared to healthy controls, and a higher concentration was found in the hypoxic patient group than in the normoxic group. Significantly higher ferritin levels were also found in COVID-19 patients, with higher values in the hypoxic group. Iron, TIBC, TSAT, RET-He, RTC, and IRF were lower, and sTfR was higher in normoxic and hypoxic patient groups than in healthy controls. UIBC and TIBC were lower, and IRF was higher in hypoxic COVID-19 patients than in normoxic patients. Compared to the normoxic patients and healthy volunteers, increased EPO concentration was found only in the hypoxic patient group. Inflammatory parameters CRP and IL-6 were both elevated in COVID-19 patients compared to the control group, and the highest concentrations were found in the hypoxic patients group. Elevated values of ferritin/hepcidin and hepcidin/iron ratios were found in the hypoxic compared to the normoxic group, while hepcidin/CRP, hepcidin/IL-6 ratios were lower in hypoxic compared to the normoxic group. No difference in hepcidin/EPO value was observed between normoxic and hypoxic patient groups.

Hepcidin concentration was positively correlated with CRP and IL-6 levels in the normoxic patient group. At the same time, a weak positive correlation with CRP and no correlation with IL-6 was found in the hypoxic patient group. Positive correlation with ferritin was observed in both patient groups, while no correlation of hepcidin with SpO_2 and EPO was found.

When patients were classified by disease severity based on prospective follow-up, hepcidin concentration was significantly higher in all patient groups than in healthy individuals. Higher hepcidin levels were found in severe and critical patients compared to mild and moderate patients. However, no significant difference was observed between severe and critical patients. Similarly, concentrations of ferritin, CRP, IL-6, and EPO were higher in severe and critical

patient groups compared to the mild and moderate group. No significant difference was found between disease severity groups for iron concentration, TSAT, sTfR, RET-He, and RTC. UIBC, TIBC, hepcidin/CRP and hepcidin/IL-6 were lower, and IRF, ferritin/hepcidin and hepcidin/iron values were higher in severe and critical patients compared to mild and moderate patient group. IL-6 and EPO concentration and hepcidin/EPO value significantly differed between severe and critical groups of patients.

The results of the univariate logistic regression analysis indicated an increased odds ratio for the development of a critical form of COVID-19 for hepcidin, EPO, CRP, IL-6, ferritin/hepcidin and hepcidin/iron ratios. On the other hand, a reduced odds ratio for developing a critical form of COVID-19 was found for UIBC and TIBC. In multiparametric logistic regression analysis, a model was obtained that included the combination of EPO and the ferritin/hepcidin ratio as parameters that proved to be the best predictors of disease severity in univariate regression analysis. The resulting model had an AUC of 0.838 (0.749 – 0.906) with 88 % correctly classified cases.

A lower total leukocyte number was found in hypoxic patients compared to normoxic COVID-19 patients and healthy controls. Also, a lower total leukocyte number was found in the mild and moderate patient groups compared with patients with severe and critical course of the disease and healthy controls. This finding is a result of the presence of neutrophilia and more pronounced lymphopenia in patients with a more severe form of the disease. In addition, a lower absolute number of monocytes and eosinophils was found in COVID-19 patients compared to healthy controls. The value of MLR was higher in COVID-19 patients, with no difference between the hypoxic and normoxic patient groups. NMR and CRP/IL-6 ratios were higher in hypoxic patients compared to normoxic patients and healthy controls, but no difference was observed between hypoxic and normoxic patient groups. All other ratios of inflammatory parameters were the highest in hypoxic COVID-19 patients, with higher values measured in normoxic patients compared to healthy controls. On the other hand, when patients were prospectively divided into groups according to the disease severity, values for MLR and CRP/IL-6 were the highest in the group of patients with a severe form of COVID-19, while values for NLR, NMR, CRP/neutrophil granulocytes, CRP/lymphocytes and CRP/monocytes were the highest and similar to each other in severe and critical patients with COVID-19. The highest values of the IL-6 ratio with leukocyte subgroups (IL-6/neutrophil granulocytes, IL-6/lymphocytes and IL-6/monocytes) were found in the critical patient group.

Conclusions: This is the first study that investigated the simultaneous presence of inflammation and hypoxia in non-anaemic COVID-19 patients selected based on rigorous

exclusion criteria to eliminate conditions and comorbidities that could influence hepcidin concentration and concentration of other iron metabolism parameters. Also, hepcidin/iron and hepcidin/EPO ratios were measured in COVID-19 patients for the first time. Hepcidin/IL-6 ratio was introduced to observe hepcidin concentration normalised to the level of inflammation. Similar results were also obtained with the hepcidin/CRP ratio that was already used in some studies. Additionally, different ratios of inflammatory parameters were calculated as it was shown that they are valuable in recognising the disease severity and predicting the outcomes of the disease. Inflammatory ratios IL-6/neutrophil granulocytes and IL-6/monocytes were applied in COVID-19 patients for the first time, while other inflammatory ratios were used previously in some studies on COVID-19 patients.

This research showed that in patients with COVID-19 the hypoxic signal was not strong enough to overcome the stimulating effect of inflammation on hepcidin concentration. The presence of more pronounced systemic inflammation probably overcame the influence of hypoxia on hepcidin concentration, which can explain elevated hepcidin concentration in hypoxic compared to normoxic COVID-19 patients. However, hepcidin concentration in hypoxic patients was not proportional to the level of systemic inflammation. The positive correlation of hepcidin with IL-6 observed in normoxic patients was not present in the group of hypoxic subjects. This study provides insight into the hepcidin concentration in different clinical settings where antagonistic signals that regulate hepcidin concentration are simultaneously present. The results of this doctoral thesis contribute to a better understanding of the complex relationships between hepcidin and other parameters of iron metabolism, hypoxia, inflammation, and erythropoiesis. However, the challenge for the future is to apply the obtained results for potential new diagnostic, prognostic, and/or therapeutic purposes not only in SARS-CoV-2 virus infection but also in various other infectious and inflammatory conditions and diseases.

Keywords: COVID-19, hepcidin, iron, ferritin, erythropoietin, interleukin 6, hypoxia, inflammation