

**SERUM CONCENTRATION OF HEPCIDIN AS AN INDICATOR  
OF IRON RESERVES IN CHILDREN**

## KONCENTRACIJA SRUMSKOG HEPCIDINA KAO INDIKATORA REZERVU GVOŽĐA U DECE

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**Summary**

**Background:** Anemia represents a significant cause of maternal and perinatal mortality, as well as child mortality. The aim of the research was to determine the serum concentration of hepcidin in children aged 6 months to 2 years and adolescents aged 11 to 19 years which suffer from iron deficiency anemia and compare it with the serum concentration of hepcidin in the control groups, as well as to determine its connection with the parameters of the iron metabolism.

**Methods:** The research included 173 examinees, 89 of them suffered from iron deficiency anemia and 84 did not suffer from iron deficiency anemia (the latter represented the control group). Blood samples were collected from all study participants. The samples were analyzed for complete blood count and parameters of iron metabolism. ELISA method was used for establishing serum hepcidin levels.

**Results:** The research showed that the concentration of hepcidin is statistically lower in children (4.4 ng/mL) and adolescents (4.1 ng/mL) who suffer from iron deficiency anemia in comparison with the control group (14 ng/mL, 10 ng/mL, respectively). The positive correlation between serum hepcidin level and iron in the serum, ferritin, the

**Kratak sadržaj**

**Uvod:** Anemija predstavlja značajan uzrok maternalnog i perinatalnog mortaliteta, kao i mortaliteta dece. Cilj istraživanja je bio da se odredi serumska koncentracija hepcidina u dece uzrasta od 6 meseci do 2 godine i adolescenata uzrasta od 11 do 19 godina koji boluju od sideropenijske anemije i uporedi sa serumskom koncentracijom hepcidina u kontrolnim grupama, kao i da se odredi njegova povezanost sa parametrima koji ukazuju na metabolizam gvožđa.

**Metode:** Istraživanjem je obuhvaćeno 173 ispitanika, 89 ispitanika koji su bolovali od sideropenijske anemije i 84 koji nisu bolovali od sideropenijske anemije (koji su predstavljali kontrolnu grupu). Krv je uzorkovana svim ispitanicima. Iz uzoraka je određivana kompletna krvna slika i vrednosti parametara metabolizma gvožđa. Nivo hepcidina u serumu je određivan ELISA metodom.

**Rezultati:** Istraživanjem je pokazano da je koncentracija hepcidina statistički značajno niža kod dece (4,4 ng/mL) i adolescenata (4,1 ng/mL) koji boluju od sideropenijske anemije u poređenju sa kontrolnom grupom (14 ng/mL, odnosno 10 ng/mL, po redosledu navođenja). Potvrđena je pozitivna korelacija između koncentracije hepcidina i gvožđa u serumu, feritina, srednjeg volumena eritrocita i

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*List of abbreviations:* ANN, Artificial neural network; ANOVA, Analysis of variance; CRP, C-reactive protein; Hb, Hemoglobin; Fe, Iron; FOP, First order polynomial model; IDA, Iron deficiency anemia; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; PLT, Platelets; RBC, Red blood cells; Rtc, Reticulocytes; TIBC, Total iron binding capacity; Trans, Transferrin; TSAT, Transferrin saturation; UIBC, Unsaturated iron binding capacity; WBC, white blood cells; WHO, World Health Organization.

mean corpuscular volume and transferrin saturation was confirmed, but the negative one occurred in serum hepcidin level, transferrin and reticulocytes.

**Conclusions:** The age of the examinees does not influence the level of serum hepcidin which makes it a more sensitive indicator of the level of iron in the body. Besides this, serum hepcidin is a reliable biological marker for the assessment of iron deficiency anemia.

**Keywords:** Anemia, hepcidin, iron deficiency, mathematical modelling

## Introduction

Anemia represents a significant cause of maternal and perinatal mortality, as well as child mortality. Besides, it is also the cause of low birth weight in newborns and leads to psychomotor developmental delay, increases susceptibility to infections and negatively reflects on the economic development of a country due to decreased productivity of the working population. The prevalence of anemia is inversely proportional to the economic development of the country (1).

Iron deficiency represents the major cause of anemia (1–3). It is considered that 50% of anemia cases in developing countries are caused by iron deficiency (1). According to the World Health Organization (WHO) estimate 47% of preschool children, as well as 25% of school children suffer from iron deficiency anemia (4). Children aged from 6 to 24 months (5) and adolescents (6) represent vulnerable groups. The incidence of anemia in adults in Vojvodina region is 7.7%, while data about incidence of anemia in children population is sparse (7).

The most important regulatory role in the iron metabolism is played by hepcidin which makes it a useful biological marker for diagnosing and following iron metabolism disorder (8–10). It functions in the way that it binds to ferroportin (FPN), the only known cell transporter of iron, which is present on the basolateral membrane of erythrocytes, hepatocytes, macrophages and placenta syncytiotrophoblasts (11, 12). FPN represents the product of SLC 40A1 gene (13) and consists of 571 amino acids (14). By binding to FPN, hepcidin induces its internalization and degradation, thus reducing intestinal iron absorption and iron release from macrophages (15–17).

So far, there has been no sufficient data that would enable a precise definition of reference values and potential cutoff values which could be a reliable indicator of iron deficiency (18). The number of studies that try to define referential values of hepcidin in children is quite small, and the obtained results are non-homogeneous (19–22).

The objective of this research was to determine the serum concentration of hepcidin in children aged 6 months to 2 years and adolescents aged 11 to 19

saturation of transferrin, a negative one between hepcidin and transferrin, as well as reticulocytes.

**Zaključak:** Uzrast ispitanika ne utiče na koncentraciju hepcidina u serumu što ga čini senzitivnijim pokazateljem nivoa gvožđa u organizmu. Osim toga, vrednost hepcidina u serumu predstavlja pouzdan biološki marker u postavljanju dijagnoze sideropenijske anemije.

**Ključne reči:** anemija, deficit gvožđa, hepcidin, matematičko modeliranje

who suffer from iron deficiency anemia (IDA) and in children of the same age group without IDA. This study aims to establish the correlation of hepcidin concentration and serum iron concentration, ferritin concentration, transferrin concentration, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and transferrin saturation (TSAT).

## Materials and Methods

The research was conducted in the form of a cross-sectional study at the Institute for Child and Youth Health Care of Vojvodina in cooperation with the Medical Laboratory of the Clinical Center of Vojvodina. The selection of subjects and sampling of blood specimens was performed during 2014 and 2015. The study was approved by the Ethics Committee of the Institute for Child and Youth Health Care of Vojvodina, and conducted in compliance with the Helsinki Declaration.

The research included the total of 173 subjects. All of them were divided into four groups. The first group consisted of 56 children aged 6 months to 2 years who had IDA, defined as Hb value lower than 110 g/L (16 female and 40 male subjects). The second group consisted of 33 adolescents aged 11 to 19 who had IDA (26 subjects were females, and 7 of them were males). This study included female adolescents whose Hb value was lower than 120 g/L or male adolescents whose Hb value was lower than 130 g/L. The third and fourth groups were control groups consisting of 32 children aged 6 months to 2 years, i.e. 52 adolescents without IDA.

The study excluded patients who suffered from associated illnesses that affect Hb concentration in serum, patients in whom the therapy of IDA was initiated and who took medicines that affect iron metabolism.

After the interview with parents and caregivers of all subjects and with adolescent subjects, they signed informed patient consent. Afterwards, blood samples were drawn for the determination of a complete blood count, reticulocytes count, serum concentration of iron, ferritin, transferrin, hepcidin, TIBC, UIBC and for C reactive protein (CRP) (in order to

eliminate the influence of infection or inflammation on iron, ferritin, transferrin and hepcidin concentration).

Blood was obtained by venepuncture carried out by trained staff. Samples for hepcidin measurement were centrifuged and immediately stored at  $-70^{\circ}\text{C}$  until analysis. Complete blood count was determined by quantitative method of flow cytometry with the application of commercial sets.

Normal values of RBC in the age group 6–24 months were considered to be  $3.7\text{--}4.5 \times 10^{12}/\text{L}$ , and in the age group 11–19 they were  $4.1\text{--}4.9 \times 10^{12}/\text{L}$ . Normal values of Hb in the age group 6–24 months were considered to be 110–120 g/L, in the age group 11–19 120–140 g/L in female persons and 130–140 g/L in male persons. Normal values of MCV in the age group 6–24 months were considered to be  $70\text{--}78 \times 10^{-15}/\text{L}$ , in the age group 11–19 they were  $78\text{--}90 \times 10^{-15}/\text{L}$ , MCH in the age group 6–24 months was  $23\text{--}27 \times 10^{-12}/\text{L}$ , in the age group 11–19 it was  $25\text{--}30 \times 10^{-12}/\text{L}$  and MCHC in both age groups was 310–340 g/L. The normal WBC count was  $4\text{--}10 \times 10^9/\text{L}$ , PLT count  $140\text{--}400 \times 10^9/\text{L}$ , and Rtc count was 0.7–2.2%.

The determination of serum concentration of C-reactive protein was performed by the device Roche/Hitachi Cobas C, with the application of commercial sets of Roche Company. Serum iron level was performed photometrically by the device Roche/Hitachi Cobas C, with the application of commercial sets of Roche Company. Normal iron levels in the age group 6–24 months were considered to be 4–25  $\mu\text{mol}/\text{L}$ , and in the age group 11–19 they were 5–33  $\mu\text{mol}/\text{L}$ . TIBC was determined photometrically by the device Roche/Hitachi Cobas C, with the application of commercial sets of Roche Company. Normal TIBC value ranges in the age group 6–24 months were considered to be 48–79  $\mu\text{mol}/\text{L}$ , and in and in the age group 11–19 they were 52–102  $\mu\text{mol}/\text{L}$ . Transferrin saturation was calculated according to the following formula:  $\text{serum Fe} \times 100/\text{TIBC}$ . Cut off value for TSAT which defines IDA is <15%. Ferritin and transferrin concentrations were determined by immunoturbidimetric method on biochemical analyzer Architect c8000 with commercial sets of Abbott Company (Wiesbaden, Germany).

Normal ferritin concentration value ranges in the age group 6–24 months were considered to be 6–24  $\mu\text{g}/\text{L}$ , and in and in the age group 11–19 they were 6–40  $\mu\text{g}/\text{L}$ . Normal transferrin concentration value ranges in the age group 6–24 months were considered to be 2.18–3.47 g/L, and in and in the age group 11–19 they were 2.33–4.44 g/L (10). Hepcidin serum concentration was measured in all subjects. We applied R&D (Research & Development) Quantikinine ELISA for the quantitative determination of human hepcidin. The producer neither defined nor recommended the range of reference values. Sensi-

tivity of the analysis was < 1.70 pg/mL. The precision of analysis was expressed through the coefficient of variance value (CV), with the precision within one series (intra-assay) CV were 4.3%, 3.1% and 3.2%, respectively, while for the precision among series (inter-assay), CV were even higher: 11%, 8% and 6.2%.

#### *Statistical analysis*

All data were processed statistically using the software package STATISTICA 10.0 (StatSoft Inc., Tulsa, OK, USA). Collected data have been subjected to analysis of variance (ANOVA) for the comparison of means, and significant differences are calculated according to post-hoc Tukey's HSD («honestly significant differences») test at  $p < 0.05$  level, 95% confidence limit.

#### *First order polynomial model*

According to general recommendations, prior to ANN modelling, first order polynomial (FOP) model was developed, and analysis of variance (ANOVA) was performed, in order to check the significant effect of the input variables over the output, as well as to justify the later use of the ANN model by the coefficient of determination ( $r^2$ ). The FOP model was used for estimation of the main effect of the process variables on responses. The independent variables used for modelling were age, Le, Er, Hb, Tr, MCV, MCH, MCHC, Fe, UIBC, TIBC, CRP, Ferritin, Trans, Hepcidin, Rtc and Tsat, while IDA was the response variable.

#### *Artificial neural network (ANN) modelling*

Artificial neural network (ANN) models were used for modelling. A multi-layer perceptron model (MLP) consisted of three layers (input, hidden and output). The MLP neural network learns using an algorithm called 'backpropagation'. Levenberg–Marquardt algorithm is proved to be the fastest and particularly adapted for networks of moderate size. During this iterative process, input data are repeatedly presented to the network (23). The coefficient of determination ( $r^2$ ) and SOS were used as parameters to check the performance (i.e. the accuracy) of the obtained ANNs.

#### *Sensitivity analysis*

Sensitivity analysis is a wide accepted technique which is necessary to use for studying the effects of observed input variables and also the uncertainties in obtained models and general network behaviour. On the basis of the developed ANN model, sensitivity analysis was performed in order to more precisely

define the influence of input variables on the observed outputs.

## Results

The hematological parameters, markers of iron metabolism and CRP are shown in *Table I*. As expected, hemoglobin, MCV, MCH, MCHC, iron, transferrin saturation and ferritin were significantly decreased, while reticulocytes, transferrin, UIBC and TIBC were increased in anemic subjects. RBC and CRP showed no differences among groups.

MCV and MCH, were significantly lower in both infant groups when compared to the adolescent groups. Iron, ferritin, UIBC, transferrin and TSAT were lower in infant control group when compared to the adolescent control group. Observed parameters were similar in both groups with IDA.

Hepcidin was significantly lower in anemic examinees in both infant and adolescent groups (4.4 ng/mL vs 4.1 ng/mL) when compared to the control groups (14 ng/mL and 10 ng/mL respectively). Hepcidin concentration was similar when compared between the age groups, both study and control.

In control groups, all the values of measured parameters fell within the reference range with the exception of erythrocyte count in infants and MCHC in adolescents (slightly elevated).

*Table II* shows correlation matrix for patients suffering from anemia and control group for 6–24 months and 11–19 years age groups. According to results presented in *Table II*, the serum iron concentration is in a positive correlation with the concentration of ferritin and TSAT, as well as in the negative correlation with UIBC and TIBC, the transferrin and Rtc ( $p < 0.01$ ). The serum iron concentration in a positive correlation with the concentration of hepcidin ( $p < 0.05$ ). UIBC is positively correlated with TIBC, transferrin and Rtc, while it is in negatively correlated with the concentration of ferritin and the TSAT ( $p < 0.01$ ). TIBC is in a positive correlation with the transferrin and Rtc, and in a negative correlation with the concentration of ferritin and TSAT ( $p < 0.01$ ). The concentration of ferritin is in a positive correlation with the concentration of hepcidin and TSAT, and in a negative correlation with the concentration of transferrin and Rtc ( $p < 0.01$ ). Transferrin is in a positive correlation with Rtc, and in a negative correlation with TSAT ( $p < 0.01$ ), as well as in the negative correlation with the concentration of hepcidin ( $p < 0.05$ ). The concentration of hepcidin in the serum is positively correlated with TSAT, and in a negative correlation with Rtc ( $p < 0.10$ ). Rtc is in a negative correlation with TSAT ( $p < 0.10$ ).

## FOP model and the analysis of variance

Analysis of variance (ANOVA) was conducted for obtained First order polynomial (FOP) model, and output was tested against the impact of input variables (*Table III*). ANOVA analysis revealed that the parameters Age, RBC, MCH, Transferrin and Hepcidin considerably influenced in forming of FOP model IDA calculation, statistically significant at  $p < 0.01$  level. The coefficient of determination ( $r^2$ ) for a FOP prediction model of IDA was relatively good (0.753), *Table III*, indicating that some other model (ANN model, for instance) could improve the validity of the developed model.

## Artificial neural network model

Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm, implemented in StatSoft Statistica's evaluation routine, was used for ANN modelling. The optimization procedures to minimize the error function between network and experimental outputs was used during ANN training cycle (23, 24), and the sum of squares (SOS) was evaluated according to the BFGS algorithm, to speed up and stabilize convergence of the results (25). ANN models were used to predict experimental variables, reasonably well, for a broad range of the process variables.

The predicted values were very close to the desired values in most cases, in terms of  $r^2$  value, for ANN models. SOS obtained with ANN models are of the same order of magnitude as experimental errors for outputs reported in the literature (24, 25). ANN model is complex because of the high nonlinearity of the developed system (24, 26).

The developed empirical models give a reasonable fit to data and successfully predict the IDA of the patient. The first order polynomial model showed high coefficients of determination for prediction of experimental results (0.753), while the artificial neural network model showed better prediction capabilities (overall  $r^2$  was 0.971).

Sensitivity analysis was performed in order to assess the effect of each change in the output due to the change in the input. It indicates how sensitive is the response variable calculated to the observed domain of input variables.

The influence of the input over the output variables, i.e. calculated changes of output variables for infinitesimal changes in input variables, as well as the importance of an input variable at a given point in the input space are shown in *Figure 1*. The influence of the input over the output variables, i.e. calculated changes of output variables for infinitesimal changes in input variables, as well as the importance of an input variable at a given point in the input space are shown in *Figure 1*. The obtained values corresponded to the level of experimental errors, and also showed

**Table I** Descriptive statistics of data.

Diagnose	Age	Samples	Parameter	Mean±SD	Parameter	Mean±SD
Anem.	11–19 years	33	WBC (10 <sup>9</sup> /L)	6.211±2.113 <sup>a</sup>	UIBC	68.639±12.910 <sup>a</sup>
Control	11–19 years	52		6.822±2.019 <sup>a</sup>		48.344±11.406 <sup>b</sup>
Anem.	6–24 months	56		10.547±3.834 <sup>b</sup>		70.887±17.567 <sup>a</sup>
Control	6–24 months	32		9.785±2.919 <sup>b</sup>		56.334±9.766 <sup>c</sup>
Anem.	11–19 years	33	RBC (10 <sup>12</sup> /L)	4.474±0.592 <sup>a</sup>	TIBC (μmol/L)	75.294±10.605 <sup>a,b</sup>
Control	11–19 years	52		4.689±0.465 <sup>a</sup>		62.652±9.290 <sup>c</sup>
Anem.	6–24 months	56		4.602±0.663 <sup>a</sup>		78.057±13.872 <sup>b</sup>
Control	6–24 months	32		4.746±0.495 <sup>a</sup>		69.254±8.771 <sup>a</sup>
Anem.	11–19 years	33	Hb (g/L)	96.364±16.524 <sup>b</sup>	CRP (mg/L)	0.049±0.202 <sup>a</sup>
Control	11–19 years	52		136.481±11.055 <sup>d</sup>		0.003±0.024 <sup>a</sup>
Anem.	6–24 months	56		83.982±15.409 <sup>a</sup>		1.291±7.415 <sup>a</sup>
Control	6–24 months	32		115.750±5.565 <sup>c</sup>		0.181±0.690 <sup>a</sup>
Anem.	11–19 years	33	PLT (10 <sup>9</sup> /L)	292.485±79.399 <sup>a</sup>	Ferritin (μg/L)	10.139±5.162 <sup>a</sup>
Control	11–19 years	52		217.231±53.427 <sup>b</sup>		20.429±6.721 <sup>b</sup>
Anem.	6–24 months	56		426.357±186.214 <sup>c</sup>		13.502±8.152 <sup>a</sup>
Control	6–24 months	32		345.500±105.856 <sup>a</sup>		19.859±11.326 <sup>b</sup>
Anem.	11–19 years	33	MCV (10 <sup>-15</sup> /L)	69.582±9.705 <sup>a</sup>	Transferin (g/L)	3.506±0.343 <sup>a</sup>
Control	11–19 years	52		83.588±5.264 <sup>c</sup>		2.740±0.245 <sup>b</sup>
Anem.	6–24 months	56		60.420±9.066 <sup>b</sup>		3.656±0.450 <sup>a</sup>
Control	6–24 months	32		72.919±6.517 <sup>a</sup>		3.007±0.368 <sup>c</sup>
Anem.	11–19 years	33	MCH (10 <sup>-12</sup> /L)	20.958±3.693 <sup>b</sup>	Hepcidin (ng/mL)	4.1±18 <sup>a</sup>
Control	11–19 years	52		29.385±2.196 <sup>d</sup>		10±7 <sup>b</sup>
Anem.	6–24 months	56		17.961±4.002 <sup>a</sup>		4.4±3.1 <sup>a</sup>
Control	6–24 months	32		24.381±2.636 <sup>c</sup>		14±24 <sup>b</sup>
Anem.	11–19 years	33	MCHC (g/L)	313.030±25.618 <sup>a</sup>	Rtc (%)	1.770±0.606 <sup>b</sup>
Control	11–19 years	52		349.731±28.398 <sup>b</sup>		1.152±0.410 <sup>a</sup>
Anem.	6–24 months	56		296.107±29.569 <sup>a</sup>		1.929±0.705 <sup>b</sup>
Control	6–24 months	32		336.531±33.318 <sup>b</sup>		1.317±0.268 <sup>a</sup>
Anem.	11–19 years	33	Fe (μmol/L)	6.215±4.345 <sup>a</sup>	TSAT (%)	8.791±7.320 <sup>a</sup>
Control	11–19 years	52		14.186±5.703 <sup>b</sup>		23.404±11.010 <sup>c</sup>
Anem.	6–24 months	56		4.751±3.249 <sup>a</sup>		6.998±6.646 <sup>a</sup>
Control	6–24 months	32		10.736±4.766 <sup>c</sup>		15.583±7.235 <sup>b</sup>

<sup>a-f</sup> Values with the same letter are not statistically different at the  $p < 0.05$  level (according to posthoc Tukey's HSD test). Legend: WBC – white blood cells, RBC – Red blood cells, Hb – Hemoglobin, PLT – Platelets, MCH – Mean corpuscular hemoglobin, MCHC – Mean corpuscular hemoglobin concentration, MCV – Mean corpuscular volume, Fe – Iron, UIBC – Unsaturated iron binding capacity, TIBC – Total iron binding capacity, CRP – C-reactive protein, Rtc – Reticulocytes, TSAT – Transferrin saturation.

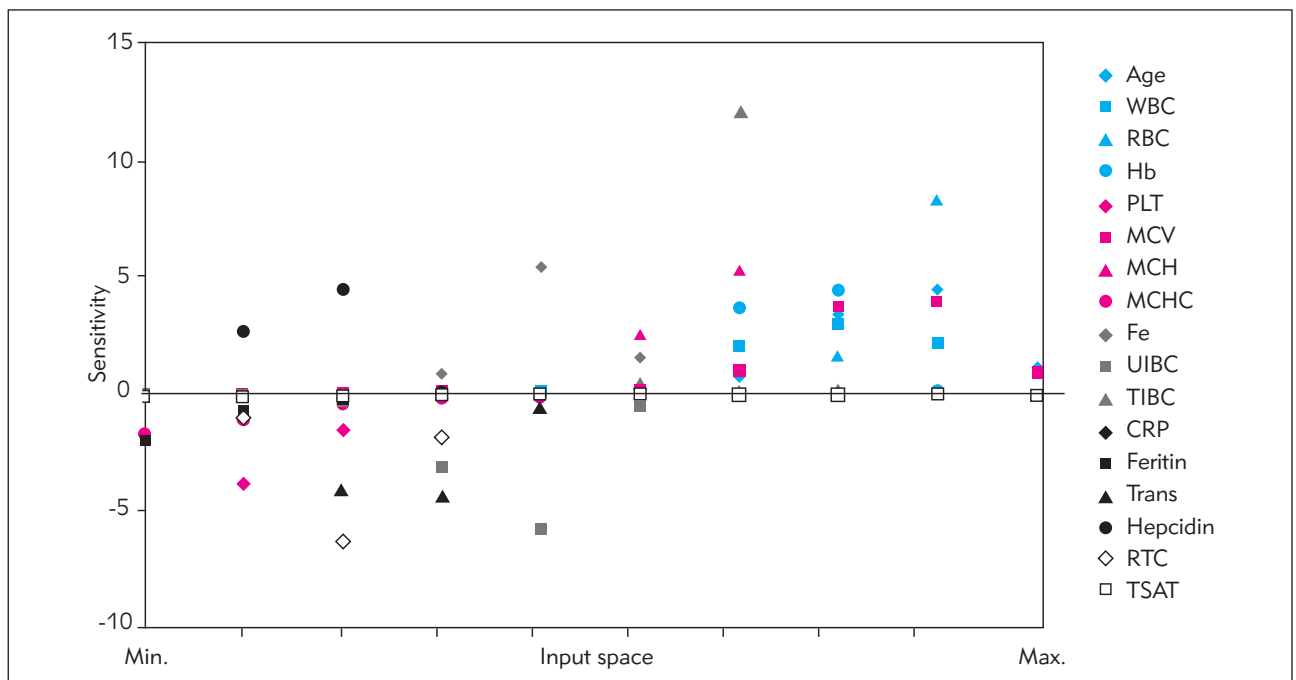


**Table II** Correlation matrix for patients suffering from anemia and control group for 6–24 months and 11–19 years age groups.

	RBC	Hb	PLT	MCV	MCH	MCHC	Fe	UIBC	TIBC	CRP	Feritin	Trans	Hepcidin	Rtc	TSAT
WBC	0.186*	-0.242 <sup>+</sup>	0.500 <sup>+</sup>	-0.313 <sup>+</sup>	-0.316 <sup>+</sup>	-0.233 <sup>+</sup>	-0.155*	0.151	0.156*	0.204 <sup>+</sup>		0.224 <sup>+</sup>			-0.154*
RBC		0.202 <sup>+</sup>		-0.157*	-0.152*						0.174*			-0.165*	
Hb			-0.460 <sup>+</sup>	0.828 <sup>+</sup>	0.885 <sup>+</sup>	0.672 <sup>+</sup>	0.753 <sup>+</sup>	-0.699 <sup>+</sup>	-0.615 <sup>+</sup>		0.521 <sup>+</sup>	-0.763 <sup>+</sup>		-0.640 <sup>+</sup>	0.736 <sup>+</sup>
PLT				-0.455 <sup>+</sup>	-0.471 <sup>+</sup>	-0.338 <sup>+</sup>	-0.342 <sup>+</sup>	0.286 <sup>+</sup>	0.230 <sup>+</sup>		-0.202 <sup>+</sup>	0.374 <sup>+</sup>		0.155*	-0.332 <sup>+</sup>
MCV					0.894 <sup>+</sup>	0.526 <sup>+</sup>	0.618 <sup>+</sup>	-0.643 <sup>+</sup>	-0.561 <sup>+</sup>		0.381 <sup>+</sup>	-0.654 <sup>+</sup>	0.183*	-0.519*	0.611*
MCH						0.781 <sup>+</sup>	0.680 <sup>+</sup>	-0.704 <sup>+</sup>	-0.654 <sup>+</sup>		0.399 <sup>+</sup>	-0.706 <sup>+</sup>	0.175*	-0.547 <sup>+</sup>	0.692 <sup>+</sup>
MCHC							0.473 <sup>+</sup>	-0.484 <sup>+</sup>	-0.490 <sup>+</sup>		0.273 <sup>+</sup>	-0.506 <sup>+</sup>		-0.446 <sup>+</sup>	0.482 <sup>+</sup>
Fe								-0.744 <sup>+</sup>	-0.550 <sup>+</sup>		0.489 <sup>+</sup>	-0.623 <sup>+</sup>	0.166*	-0.444 <sup>+</sup>	0.971 <sup>+</sup>
UIBC									0.915 <sup>+</sup>		-0.468 <sup>+</sup>	0.676 <sup>+</sup>		0.452 <sup>+</sup>	-0.819 <sup>+</sup>
TIBC											-0.409 <sup>+</sup>	0.601 <sup>+</sup>		0.409 <sup>+</sup>	-0.673 <sup>+</sup>
Feritin												-0.558 <sup>+</sup>	0.322 <sup>+</sup>	-0.328 <sup>+</sup>	0.490 <sup>+</sup>
Trans													-0.171*	0.584 <sup>+</sup>	-0.616 <sup>+</sup>
Hepcidin														-0.134**	0.144**
Rtc															-0.426 <sup>+</sup>

<sup>+</sup> Correlation is statistically significant at the  $p < 0.01$  level; \* Correlation is statistically significant at the  $p < 0.05$  level.

Legend: WBC – White blood cells, RBC – Red blood cells, Hb – Hemoglobin, PLT – Platelets, MCH – Mean corpuscular hemoglobin, MCHC – Mean corpuscular hemoglobin concentration, MCV – Mean corpuscular volume, Fe – Iron, UIBC – Unsaturated iron binding capacity, TIBC – Total iron binding capacity, CRP – C-reactive protein, Trans – Transferin, Rtc – Reticulocytes, TSAT – Transferrin saturation.



**Figure 1** Sensitivity analysis – the influence of the input over the output variables.

that Age, WBC, RBC, Hb, PLT, MCV, MCH, MCHC, Fe, UIBC, TIBC, CRP, Ferritin, Transferin, Hepcidin, Rtc and TSAT influenced on IDA.

Sensitivity analysis was used to investigate the influence of input variables on the observed outputs, evaluated at specific centile points for each input variable. The influence of the input over the output variables, i.e. calculated changes of the output variables for infinitesimal changes in the input variables are shown in *Figure 1*.

The more pronounced effect on IDA could be observed by infinitesimal changes in variable Age, closer to the maximum of input space of this variable. It means that if the Age variable is higher, it would be more likely that the IDA would be indicated. The effects of infinitesimal changes in MCV and RBC variables IDA are more indicative closer to the maximums of these input variables (within the tested input spaces, described in the descriptive statistics table). Also, the impacts of small variations in Hb, MCH and Fe on IDA determination, could be more easily noticed if they are closer to the higher levels of these variables, closer to the maximum of defined input space. The effect of infinitesimal changes in the PLT on IDA determination, could be observed closer to the maximum level of this variable. According to sensitivity analysis, variables MCHC and CRP had no significant impact on the determination of IDA, which coincide to result of ANOVA for the FOP model. The effect of infinitesimal changes in UIBC, closer to the maximum of input space influences IDA more pronounced, while the impact of small variations in variable Ferritin is more easily observed at the minimum of the input space. The effect of infinitesimal changes in variable Hepcidin, at the lower end of the input space indicates the more pronounced changes in IDA value. The effects of infinitesimal changes in Rtc closer to the maxim of the input space could be more easily indicated in IDA calculation. The variable TSAT expressed no impact on the determination of IDA, according to sensitivity analysis.

## Discussion

Hepcidin is synthesized as a response to the iron body level, inflammation, hypoxia and anemia (14, 27–29). Positive regulators of hepcidin synthesis are increased iron values, infection and/or inflammation, while negative regulators of hepcidin synthesis are iron deficiency, anemia, hypoxia and erythropoiesis (11, 19, 30, 31).

It was suggested at hepcidin concentration exhibits great inter-individual differences and that hepcidin values are influenced by gender and age, with the possibility of the existence of significant difference among populations (32, 33).

In addition to the standard parameters of iron metabolism that are used in everyday clinical practice, we measured the serum hepcidin. The tests conducted for adult population have shown different levels of hepcidin, varying for sex and age, however, the results were not consistent. Also, a small number of studies that evaluated hepcidin in children didn't propose the reference value, nor the correlation between hepcidin and sex, age, or iron level concentration in various clinical conditions (19). In the general population, hepcidin levels are significantly lower for women under 50 years of age compared to men in the same age group, and after fifty years of age is similar in both sexes and relatively constant values in the coming decades. This is due to the increased need for iron in women during the reproductive age (34).

In our study, serum hepcidin was determined by ELISA method. The resulting mean value of hepcidin in the age group of 6 to 24 months for patients suffering from anemia was 4.4 ng/mL, while this value reached 14 ng/mL for the control group. In the age group 11–19 years, for the group of patients suffering from anemia, the mean value of hepcidin was 4.1 ng/mL, while in the control group this value reached 10 ng/mL. There was no significant difference in mean hepcidin concentration observed between two age groups, but the difference was significant between both IDA age groups and corresponding controls ( $p < 0.05$ ). According to the literature, a wide range of values on the basis of measurements performed by ELISA test of hepcidin ranged between 29–254 ng/mL for healthy men, and 17–286 ng/mL for healthy women (35). In a study performed by Galesloutet al. (18), which included 2,998 healthy volunteers, it was found that hepcidin serum ranged between 4.1 nmol/L for premenopausal women and 7.8 nmol/L for men.

In our study, it was confirmed that there was no significant difference between the mean values of ferritin observed for two age groups, and there was statistically significant difference between patients suffering from anemia and control groups. Since ferritin is a protein of the acute phase of the inflammatory response its value is increased during infection and inflammation, the normal values does not exclude the existence of IDA. Therefore, the level of ferritin should be correlated with other markers of inflammation (36, 37).

Although hepcidin acts as an acute phase reactant in the inflammatory response, as well as ferritin, the hepcidin value declines more rapidly after the removal of infectious or inflammatory signal. According to Uijterschout et al. (29), for children with iron deficiency in convalescence period, hepcidin serum is decreased in order to allow the intestinal absorption of iron for erythropoiesis, while the ferritin value in this period is still above the limit value (cut off) that

is indicative for the iron deficiency. Thus, hepcidin represents a more sensitive indicator of iron deficiency than ferritin, however, the clinical use of hepcidin is being hampered by the lack of defined reference values for the specific age (23).

From *Figure 1* it can be noted that hepcidin has an important role in the definition of the neural network model. It is good predictor of iron status and it can suggest whether the patient is or is not iron deficient. This model suggests that hepcidin is far better indicator of IDA or iron deficiency than the Hb itself (Hb is not even statistically significant in the linear model). Hepcidin can be considered as a factor that indicates iron deficiency much before Hb.

It is not influenced by age which makes it a more sensitive indicator of the iron body stores. Besides this, serum hepcidin is a reliable biological marker of the iron deficiency.

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## Contribution statement

CJ and KJ conceived the idea for the study. CV, NS and SA contributed to the design of the research. All authors were involved in data collection. PL analyzed the data. BSM coordinated funding for the project. All authors edited and approved the final version of the manuscript.

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## Conflict of interest statement

The authors stated that they have no conflicts of interest.



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