

# Serum hepcidin levels and iron parameters in children with iron deficiency

Hyoung Soo Choi<sup>1</sup>, Sang Hoon Song<sup>2</sup>, Jae Hee Lee<sup>3</sup>, Hee-Jin Kim<sup>4</sup>, Hye Ran Yang<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seongnam,

<sup>2</sup>Department of Laboratory Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Seoul,

<sup>3</sup>Department of Pediatrics, Chosun University Hospital, Gwangju, <sup>4</sup>Department of Laboratory Medicine & Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

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## Background

Iron deficiency (ID) and iron deficiency anemia (IDA) are common nutritional disorders in children. Hepcidin, a peptide hormone produced in the liver, is a central regulator of systemic iron metabolism. We evaluated whether serum hepcidin levels can diagnose ID in children.

## Methods

Sera from 59 children (23 males and 36 females; 5 months to 17 years) were analyzed for hepcidin-25 by ELISA. Patients were classified according to hemoglobin level and iron parameters as: IDA (N=17), ID (N=18), and control (N=24).

## Results

Serum hepcidin, ferritin, soluble transferrin receptor (sTfR), transferrin saturation, and hemoglobin levels differed significantly between groups ( $P < 0.0001$ ). Serum hepcidin and ferritin levels (mean  $\pm$  SD) were  $2.01 \pm 2.30$  and  $7.00 \pm 7.86$ ,  $7.72 \pm 8.03$  and  $29.35 \pm 24.01$ ,  $16.71 \pm 14.74$  and  $46.40 \pm 43.57$  ng/mL in the IDA, ID, and control groups, respectively. The area under the receiver operating characteristic curve for serum hepcidin as a predictor of ID was 0.852 (95% CI, 0.755–0.950). Hepcidin  $\leq 6.895$  ng/mL had a sensitivity of 79.2% and specificity of 82.8% for the diagnosis of ID. Serum hepcidin levels were significantly correlated with ferritin, transferrin saturation, and hemoglobin levels and significantly negatively correlated with sTfR level and total iron binding capacity ( $P < 0.0001$ ).

## Conclusion

Serum hepcidin levels are significantly associated with iron status and can be a useful indicator of ID. Further studies are necessary to validate these findings and determine a reliable cutoff value in children.

**Key Words** Serum hepcidin, Iron deficiency, Children

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## Correspondence to

Hye Ran Yang, M.D., Ph.D.  
Department of Pediatrics, Seoul National University Bundang Hospital, 82, Gumi-ro 173 Beon-gil, Bundang-gu, Seongnam 463-707, Korea  
Tel: +82-31-787-7285  
Fax: +82-31-787-4054  
E-mail: hryang@snuhb.org

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## INTRODUCTION

Iron deficiency (ID) and iron deficiency anemia (IDA) are common nutritional disorders in children that place children at risk of impaired psychomotor and/or mental development [1]. However, commonly used tests of iron status have limitations. Ferritin is an indicator of iron stores, but its levels are elevated in patients with coexisting inflammation. Soluble transferrin receptor (sTfR) levels reflect tissue ID, but they are influenced by erythropoietic activity [2]. In

addition, transferrin saturation level may be affected by inflammation and undergoes diurnal variation [1].

Hepcidin-25, a 25-amino acid peptide hormone produced in the liver, is a central regulator of systemic iron metabolism [3, 4]. Hepcidin downregulates duodenal iron absorption and macrophage iron release by modulating cellular iron export via ferroportin [5]. The dysregulation of hepcidin production is associated with a variety of iron disorders [6]. Hepcidin deficiency is the cause of iron overload in hereditary hemochromatosis, while hepcidin excess is associated with anemia of inflammation, chronic kidney disease, and



iron-refractory iron deficiency anemia [6, 7].

Hepcidin levels are reduced in patients with ID. Therefore, measurement of blood or urine hepcidin levels may enable the determination of iron requirements and be an accurate indicator of physiological ID [4]. The use of serum hepcidin level as an index for ID has been tested in adult populations [8-11]. However, very few studies have investigated the effectiveness of serum hepcidin measurements in children [12-14]. Furthermore, the sensitivities and specificities of various serum hepcidin cutoff levels in the diagnosis of ID have not been determined in either adults or children.

This study evaluated the use of serum hepcidin levels as a diagnostic test of ID in children. To this end, the correlations between serum hepcidin levels and other iron parameters were determined. The sensitivity and specificity of serum hepcidin as an indicator of ID were determined using receiver operating characteristic (ROC) curves.

## MATERIALS AND METHODS

### 1. Subjects

In total, 59 children (23 males and 36 females) were enrolled in this study. The age of the patients ranged from 5 months to 17 years (median, 4 years). Venous blood samples were drawn, and serum samples were frozen at  $-70^{\circ}\text{C}$  until needed for the hepcidin-25 assay. The patients were classified according to hemoglobin level and iron parameters as follows: Group 1, IDA (N=17); Group 2, ID without anemia (N=18), and Group 3, controls with normal iron levels (N=24).

### 2. Iron parameters

Serum was analyzed for ferritin, iron, total iron binding capacity (TIBC), transferrin saturation (Iron/TIBC), and C-reactive protein (CRP) levels. sTfR level was measured by a competitive ELISA (C-ELISA) (R&D Systems, Inc., Minneapolis, MN, USA). ID was defined as serum ferritin  $<12$  ng/mL in children aged  $\leq 5$  years or  $<15$  ng/mL in children  $>5$  years, or as transferrin saturation  $<16\%$  [1]. IDA was defined as a significantly reduced hemoglobin level and decreased mean corpuscular volume (MCV) with ID [1].

### 3. Serum hepcidin measurement

Serum hepcidin-25 levels were measured by a C-ELISA using a commercial kit from Peninsula Laboratories (Bachem, Torrance, CA, USA) as described previously [15, 16]. Patients' samples were assayed in duplicate. The results from the C-ELISA were compared with those of the standard curves developed from calibrators run simultaneously with study samples.

### 4. Statistical analysis

SPSS version 19 was used for data analysis. All values are expressed as mean $\pm$ SD. The differences between study groups were tested by the nonparametric Kruskal-Wallis test. Correlations between variables were calculated using

Pearson's correlation analysis for numerical data. The diagnostic utility of serum hepcidin level as a test for ID was evaluated according to the area under the ROC curve (AUC). For Pearson's correlation and linear regression modeling, the log-transformed values of hepcidin and ferritin were used to stabilize variances and satisfy normality assumptions. Linear regression analyses were used to evaluate the effects of age-, gender-, and body mass index (BMI)-adjusted independent variables on log hepcidin levels as continuous dependent variables. The level of significance was set at  $P < 0.05$ .

### 5. Ethics

Informed consent was obtained from all subjects and/or their guardians. This study was approved by the Institutional Review Board of the Seoul National University Bundang Hospital.

## RESULTS

### 1. Study population characteristics

The characteristics of the 59 subjects enrolled in this study are shown in Table 1. There were no significant differences in the clinical characteristics between the 3 groups with respect to age, gender, or BMI ( $P > 0.05$ ). In addition, CRP level, time of sampling, and the proportion of the patients with *Helicobacter pylori* infection did not significantly differ between groups.

### 2. Hematologic data and iron parameters

There were significant differences in the levels of serum hepcidin, ferritin, sTfR, iron, transferrin, transferrin saturation, and hemoglobin between the 3 groups ( $P < 0.0001$ , Kruskal-Wallis test). Serum hepcidin levels in Group 1 ( $2.01 \pm 2.30$ ; range, 0.40–5.30 ng/mL) and Group 2 ( $7.72 \pm 8.03$ ; range, 1.26–23.37 ng/mL) were significantly lower than that in Group 3 ( $16.71 \pm 14.74$ ; range, 3.24–66.86 ng/mL) (Table 1).

### 3. Serum hepcidin level as an indicator of ID and IDA

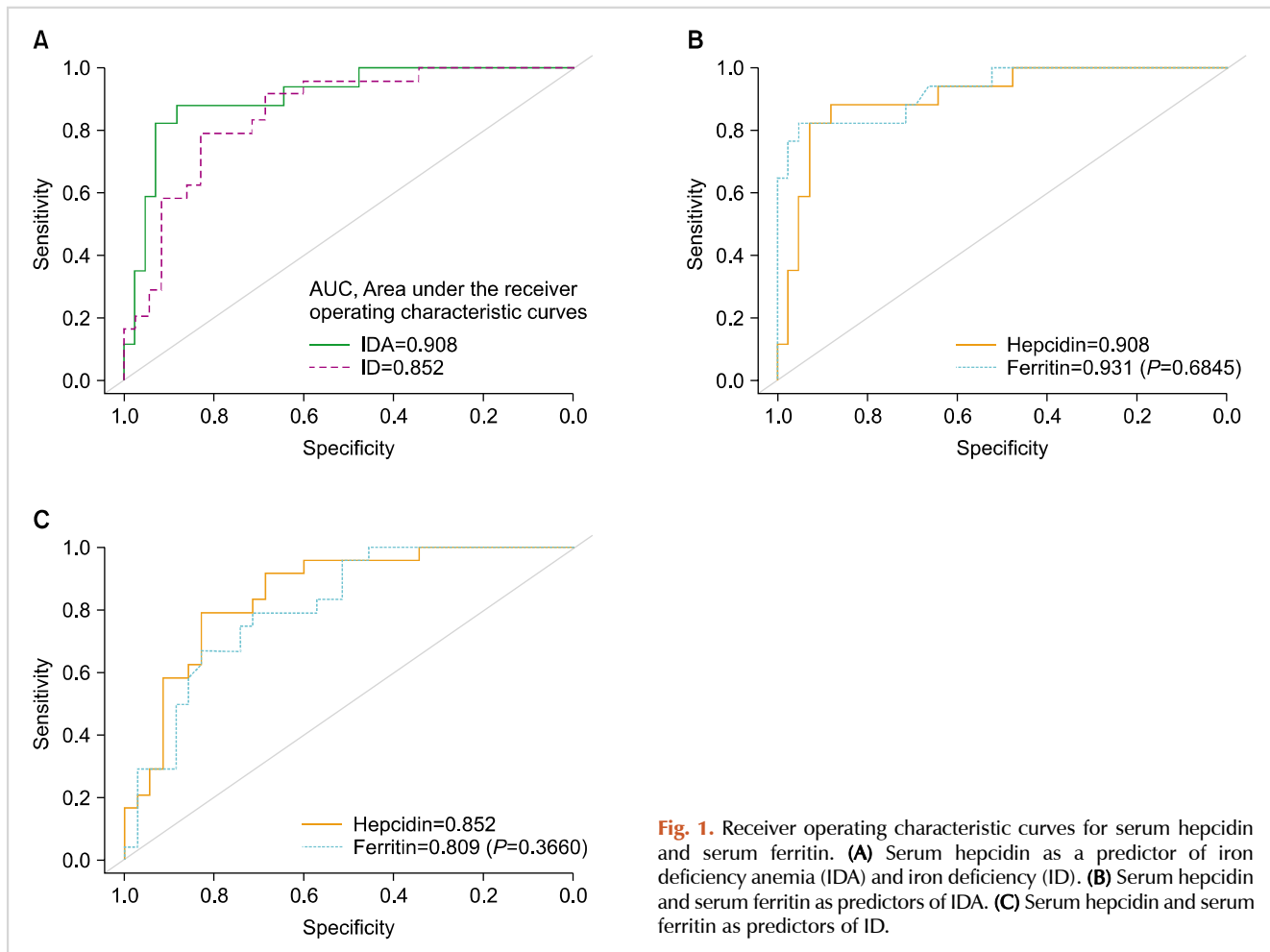
The ROC curves for serum hepcidin and serum ferritin as predictors of ID and IDA are shown in Fig. 1. The AUC of serum hepcidin was 0.852 (95% CI, 0.755–0.950) for Group 2 and 0.908 (95% CI, 0.824–0.991) for Group 1. The AUC of serum ferritin was 0.809 (95% CI, 0.700–0.918) for Group 2 (ID group) and 0.931 (95% CI, 0.857–1.000) for Group 1 (IDA group); the AUC values for serum ferritin in the 2 groups did not significantly differ from those for serum hepcidin ( $P = 0.3660$  and  $P = 0.6845$ , respectively). A scatter plot of the distribution of serum hepcidin levels around the cutoff point in the 3 groups is shown in Fig. 2. Serum hepcidin  $\leq 6.895$  ng/mL had a sensitivity of 79.2% and specificity of 82.8% for Group 2. Meanwhile, serum hepcidin  $\leq 2.735$  ng/mL had a sensitivity of 88.1% and specificity of 88.2% for Group 1.

**Table 1.** Subject characteristics.

	Group 1 (N=17)	Group 2 (N=18)	Group 3 (N=24)	P
Age (months)	101.2±70.34	52.83±52.94	67.25±58.01	0.127
Male/female	7/10	8/10	8/16	0.747
BMI (kg/m <sup>2</sup> )	16.80±5.10	17.17±3.03	15.84±4.48	0.258
Hepcidin (ng/mL)	2.01±2.30	7.72±8.03	16.71±14.74	<0.0001
Ferritin (ng/mL)	7.00±7.86	29.35±24.01	46.40±43.57	<0.0001
sTfR (nmol/L)	4.63±22.67	35.16±11.59	27.25±10.63	<0.0001
Transferrin saturation (%)	4.44±2.67	11.87±6.02	23.80±9.48	<0.0001
Iron (µg/dL)	20.18±11.90	45.61±18.72	82.29±28.31	<0.0001
TIBC (µg/dL)	466.41±66.17	395.28±48.62	342.38±103.48	<0.0001
Hb (g/dL)	8.26±1.78	12.26±0.98	12.76±1.05	<0.0001
CRP (mg/dL)	0.13±0.22	0.16±0.46	0.19±0.48	0.566
Time of sampling (1/2/3)	10/6/1	9/6/3	10/13/1	0.403
<i>H. pylori</i> (yes/no)	3/14	1/17	0/24	0.084

Time of sampling (1/2/3): 1, before midday; 2, midday to 5 pm; 3, after 5 pm.

Abbreviations: BMI, body mass index; sTfR, soluble transferrin receptor; TIBC, total iron binding capacity; Hb, hemoglobin; CRP, C-reactive protein; *H. pylori*, *Helicobacter pylori*.

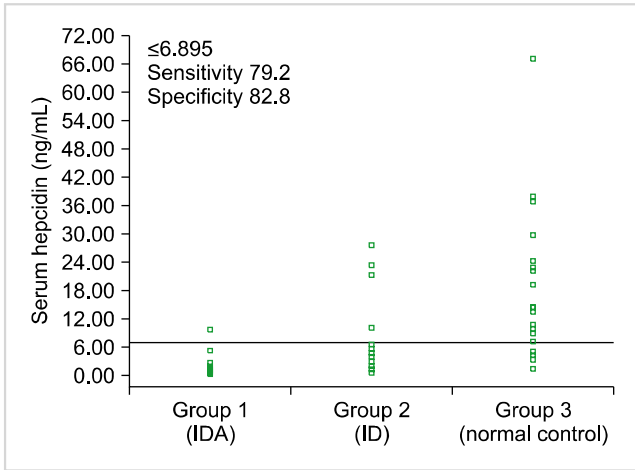


**Fig. 1.** Receiver operating characteristic curves for serum hepcidin and serum ferritin. **(A)** Serum hepcidin as a predictor of iron deficiency anemia (IDA) and iron deficiency (ID). **(B)** Serum hepcidin and serum ferritin as predictors of IDA. **(C)** Serum hepcidin and serum ferritin as predictors of ID.

#### 4. Factors associated with serum hepcidin levels

Serum hepcidin levels were significantly positively correlated with the levels of ferritin, iron, transferrin saturation, and hemoglobin ( $P < 0.05$ ) (Table 2). Meanwhile, serum hepcidin levels were significantly negatively correlated with

sTfR and TIBC ( $P < 0.0001$ ) (Table 2). Among these parameters, log ferritin showed the strongest positive correlation with log hepcidin ( $P < 0.0001$ ) (Fig. 3). Three IDA patients and 1 ID patient with *H. pylori* infection showed compatible low serum hepcidin levels. Adjusted linear regression analy-

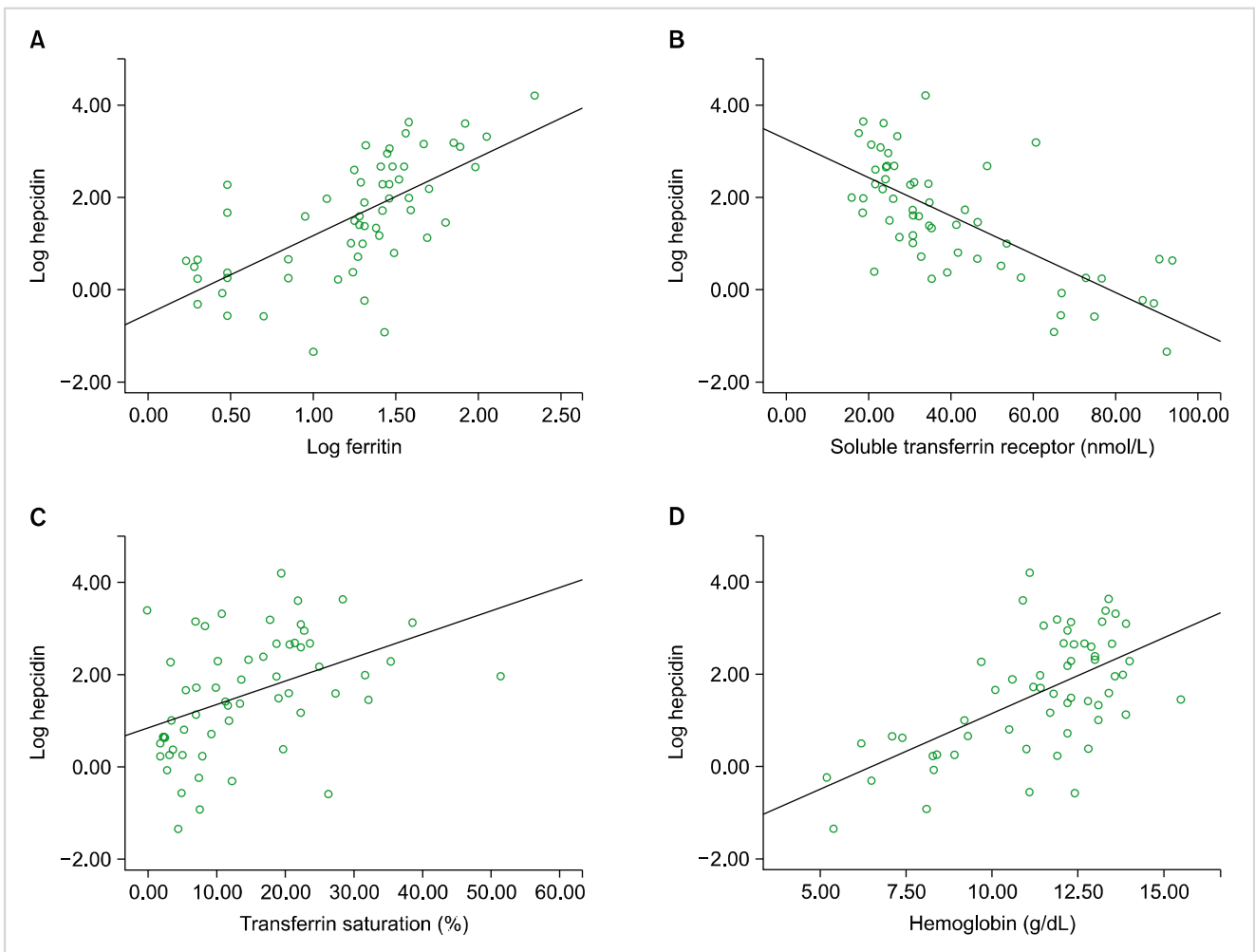


**Fig. 2.** Distribution of serum hepcidin levels in the IDA, ID, and normal controls around the cutoff point. IDA, iron deficiency anemia; ID, iron deficiency.

**Table 2.** Correlations between log hepcidin and other parameters.

	r	P
Age	-0.204	0.121
Gender	-0.073	0.584
BMI	-0.103	0.436
Ferritin	0.602	<0.0001
Log ferritin	0.666	<0.0001
sTfR	-0.712	<0.0001
Transferrin saturation	0.429	0.001
Iron	0.366	0.004
TIBC	-0.563	<0.0001
Hb	0.604	<0.0001
CRP	0.185	0.161
Time of sampling	0.091	0.492
Relapse	-0.265	0.043
<i>H. pylori</i> infection	-0.284	0.029

Abbreviations: BMI, body mass index; sTfR, soluble transferrin receptor; TIBC, total iron binding capacity; Hb, hemoglobin; CRP, C-reactive protein; *H. pylori*, *Helicobacter pylori*.



**Fig. 3.** Correlations between serum hepcidin and other parameters. Common regression lines are shown. (A) Log-transformed serum hepcidin and log-transformed ferritin ( $r=0.666$ ). (B) Log-transformed serum hepcidin and soluble transferrin receptor ( $r=-0.712$ ). (C) Log-transformed serum hepcidin and transferrin saturation ( $r=0.429$ ). (D) Log-transformed serum hepcidin and hemoglobin ( $r=0.604$ ).

**Table 3.** Adjusted<sup>a)</sup> simple linear regression for log hepcidin.

	$\beta$	95% CI	SE	P
Ferritin	0.021	0.013, 0.028	0.004	<0.0001
Log ferritin	1.579	1.066, 2.091	0.256	<0.0001
sTfR	-0.039	-0.051, -0.028	0.006	<0.0001
Transferrin saturation	0.053	0.026, 0.079	0.031	<0.0001
Iron	0.015	0.006, 0.024	0.004	0.001
TIBC	-0.007	-0.010, -0.005	0.001	<0.0001
Hb	0.313	0.201, 0.424	0.056	<0.0001
CRP	0.342	-0.488, 1.172	0.414	0.412

<sup>a)</sup>Adjusted for age, gender, and body mass index.

Abbreviations: sTfR, soluble transferrin receptor; TIBC, total iron binding capacity; Hb, hemoglobin; CRP, C-reactive protein; CI, confidence interval; SE, standard error.

ses demonstrated that log hepcidin was positively associated with ferritin, iron, transferrin saturation, and hemoglobin and negatively associated with sTfR and TIBC (Table 3).

## DISCUSSION

Since its discovery, many clinical applications for hepcidin measurement have been proposed, including its use in the diagnosis of anemia of inflammation, anemia associated with chronic kidney disease and hemodialysis, genetic hemochromatosis, and ID [10]. However, how hepcidin measurements complement the existing array of iron indices remains unclear [10].

Hepcidin is encoded as an 84-amino acid prepropeptide [17], which is cleaved to yield the 60-amino acid form called prohepcidin, which is further processed to yield the 25-amino acid form of hepcidin [17]. Previous studies investigating the use of prohepcidin as a marker for ID failed to find significant correlations between prohepcidin level and iron measurements in children [18, 19]. However, levels of the mature bioactive form of hepcidin-25 are correlated with iron status and erythropoiesis [20].

Immunochemical and mass spectrometric assays are available to measure the levels of hepcidin in serum, plasma, and urine; moreover, an international effort is underway to standardize these assays [7, 21]. A comparative study of different assays for hepcidin analysis found that although the absolute values of the results in each laboratory differ significantly, the results of samples within a given laboratory are well correlated between assays and analytical variance is generally low [21]. The development of reference hepcidin preparations would enable inter-laboratory comparison of assays and the standardization of units and reference ranges, facilitating the clinical use of a hepcidin index [21, 22].

This study evaluated the value of serum hepcidin levels as a diagnostic test for ID and IDA in children. Serum hepcidin levels measured by C-ELISA were significantly lower in children with ID and IDA than that in children in the normal control group and were more profoundly reduced in IDA. Furthermore, serum hepcidin levels were correlated with iron status in all groups. Positive correlations were

found between serum hepcidin level and serum ferritin, iron, transferrin saturation, and hemoglobin; conversely, negative correlations were found with sTfR and TIBC. Among these parameters, log ferritin was most strongly positively correlated with log hepcidin. These results are concordant with those of previous reports [23, 24], indicating that urinary hepcidin is positively associated with hemoglobin, MCV, iron, ferritin, and transferrin saturation. Another study [12] reports similar results with serum hepcidin from cord blood.

The mean serum hepcidin level in normal control children in this study was 16.71±14.74 ng/mL. Mean serum hepcidin levels were significantly lower in the ID (7.72±8.03 ng/mL) and IDA groups (2.01±2.30 ng/mL). Low serum hepcidin in ID and IDA can be attributed to the lower total iron stores in these groups than that in the normal controls [13]. Serum hepcidin levels in this study were lower than the reference range reported in healthy male and female adults (median and 5–95% reference range, 112 ng/mL [29–254 ng/mL] for men and 65 ng/mL [17–286 ng/mL] for women) [8]. In addition, the reference range in 137 neonate cord blood hepcidin samples was 20.5–231.9 ng/mL (median, 78.4 ng/mL) [12], which is higher than that in the present study. Therefore, it is necessary to establish a reference range of serum hepcidin levels for children other than neonates.

We determined 2 cutoff points for serum hepcidin to differentiate children with ID and IDA from normal controls. These 2 cutoff points had narrow confidence intervals and favorable predictive potentials. Hepcidin ≤2.735 ng/mL had a sensitivity of 88.1% and specificity of 88.2% for diagnosing IDA. Meanwhile, hepcidin ≤6.895 ng/mL had a sensitivity of 79.2% and specificity of 82.8% for diagnosing ID. In 261 premenopausal female blood donors (with a 95% reference range of serum hepcidin levels from 8.2–199.7 ng/mL), serum hepcidin <8 ng/mL had a sensitivity of 41.5% and specificity of 97.6% for diagnosing ID, while <18 ng/mL had a sensitivity of 79.2% and specificity of 85.6% [10]. More studies are required to determine a reliable cutoff value of serum hepcidin in ID.

In general, while AUC values ≤0.75 are not clinically useful, AUC values ≥0.97 have high clinical value; these values correspond to likelihood ratios of approximately 10 and 0.1, respectively [25]. The AUC of serum hepcidin for

the detection of IDA is reported to be 0.89 in non-anemic female blood donors [10]. Meanwhile, the AUC for serum hepcidin in the present study was 0.852 for the detection of ID and 0.908 for the detection of IDA, demonstrating moderate discriminatory power. These values are comparable to the AUC of serum ferritin determined in the present and previous studies [26, 27]. Therefore, the results suggest serum hepcidin has potential utility as a diagnostic test for ID in children.

Hepcidin production is induced by inflammation and iron overload and is suppressed in patients with ID, hypoxia, anemia, and conditions characterized by increased erythropoietic activity [2, 7]. Serum hepcidin levels are positively correlated with CRP levels [12]. Patients with signs of infection or inflammation were excluded from the present study. Therefore, we did not find a significant correlation between serum hepcidin and CRP levels. Four patients in the present study with *H. pylori* infection had IDA or ID and compatible low serum hepcidin levels. These results are concordant with those of Cherian *et al.* [23], who report no differences in the urinary hepcidin levels of children regardless of *H. pylori* seropositivity. One possible explanation for this is that *H. pylori*-induced inflammation does not affect iron status via increased hepcidin production in children [23]. Therefore, it is necessary to evaluate the value of serum hepcidin for diagnosing ID under inflammatory conditions. Patients with anemia of inflammation and IDA have significantly lower hepcidin levels than patients with only anemia of inflammation [9, 28].

Hepcidin levels can be measured in the serum, plasma, and urine. Initial studies investigating the role of hepcidin in iron pathophysiology used urinary hepcidin assays for technical reasons [8]. The urinary hepcidin assay provides an indirect measure of the circulating hormone level and allows development of a potential non-invasive means for diagnosing ID, which could be particularly useful for children [13, 23, 24]. However, the higher pre-analytical variability associated with urine specimens compared to serum is a potential limitation of this approach [29]. Further evaluation of urine hepcidin for non-invasive monitoring of iron status in children is necessary.

Diurnal variation is a potential limitation of the use of serum hepcidin levels for diagnostic tests [29, 30]. Serum hepcidin levels exhibit diurnal variations similar to those of serum iron levels, with levels at noon and 8 pm being significantly higher than those at 8 am [8]. However, we did not identify a significant difference in serum hepcidin levels between samples taken in the morning and afternoon.

The small number of subjects analyzed is a limitation of this study. The cutoff values of serum hepcidin for diagnosing ID determined in the present study should be confirmed in studies with larger sample sizes. However, to our knowledge, this is the first trial to determine a cutoff level for serum hepcidin level for the diagnosis of ID in children.

In conclusion, serum hepcidin levels are significantly associated with iron status in children, and could be useful indicators of ID. Further studies are necessary to confirm the

value of serum hepcidin measurement in the diagnosis of ID and to determine the reliable reference range and cutoff values in children.

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