Immunoassay-Based Serum Hepcidin Reference Range Measurements in Healthy Children: Differences Among Age Groups

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Background: Hepcidin is a peptide hormone that plays a key role in regulating iron absorption from the small intestine and body iron distribution. Alterations in hepcidin concentrations have been associated with chronic inflammatory conditions or inherited diseases of iron metabolism. The aim of our study was to evaluate healthy children in order to define normal reference range of serum hepcidin concentrations. The universal use of a reliable commercial ELISA kit gives the ability to compare our results with those from previous studies. **Methods:** We evaluated 180 healthy children (88 boys, mean age: 67.55 ± 39.26 months, median: 60, range: 24–156 months) aged 2–12 years, using an immunoassay kit. **Results:** Hepcidin median values were 46.94 ng/ml for boys and 46.79 ng/ml for girls. No significant differences were observed between

boys and girls. There seem to be significantly higher values of hepcidin in older children (10–12 years old). This trend was constant and statistically significant in boys after gender and age group stratification. Although this trend was more prominent in girls, it was not statistically significant. **Conclusions:** This study aims at setting up reference values for serum hepcidin concentrations in healthy pediatric population by using a well-established laboratory kit. The difference in hepcidin concentrations in older children could be attributed to different growth rates. Additionally, differences between values in adults and children could reflect alterations in iron metabolism between those two age groups. J. Clin. Lab. Anal. 29:10–14, 2015. © 2014 Wiley Periodicals, Inc.

Key words: hepcidin; pediatrics; iron metabolism; ELISA; ferritin

BRIEF REPORT

Over the last decade, a significant progress was noted in clarifying the iron-metabolism pathway. The isolation of the new molecule “hepcidin” among the well-established iron parameters raised great investigational attention as it plays a key role in iron metabolism. Hepcidin is initially synthesized as 84 amino acids peptide (prohepcidin) that

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Received 11 June 2013; Accepted 31 October 2013

DOI 10.1002/jcla.21719

Published online in Wiley Online Library (wileyonlinelibrary.com).

is converted to its bioactive form, 25 amino acids peptide, by hepatocytes, secreted into the bloodstream, and cleared from the circulation by the kidneys. It regulates the levels of circulating iron by controlling the absorption of dietary iron and the release of iron from macrophages and hepatocytes. Except hepatocytes, other cell types including macrophages and adipocytes also contain hepcidin mRNA but their contribution to the production of bioactive hepcidin has not been established yet (1).

Hepcidin synthesis is under control of both physiologic and pathologic mechanisms. The need for increased iron in peripheral blood leads in decrease of hepcidin concentration in circumstances of increased erythropoietic activity such as hypoxia, iron deficiency, and anemia or in cases of ineffective erythropoiesis. The decrease of hepcidin results in increased absorption of iron from the intestine and increased release from iron-storing cells by binding internalization and degradation of the cellular iron efflux molecule, ferroportin (2–5). On the contrary, infection, chronic inflammation, and iron overload result in increase of hepcidin concentration and subsequent decrease in circulating iron with the exception of hereditary hemochromatosis where a mutated hepcidin gene results in deficient excretion of the molecule leading to iron accumulation (6).

The important role of hepcidin in iron homeostasis raised a great investigational interest over the last years. Starting from research on mice and extrapolating the results to humans, hepcidin is a helpful tool in rare genetic diseases such as hereditary hemochromatosis as it is negatively correlated with the severity of iron overload. Additionally, its role is important in the diagnosis of iron refractory iron deficiency anemia (IRIDA) as well as in anemia of chronic disease (7). Hepcidin concentrations may also be helpful in predicting erythropoietin response and guiding treatment with intravenous iron and erythropoietin, especially in patients with chronic kidney disease (8). It may be helpful in defining which patients could benefit from oral iron therapy as studies of healthy adults have shown increased correlation between hepcidin concentration and radiolabeled iron absorption (9). Finally, the application of serum hepcidin measurements in monitoring novel therapies with agents that target the molecule of hepcidin or ferroportin could be of significant importance in disorders concerning the iron metabolism.

Immunochemical and mass spectrometric assays of serum hepcidin are available for research purposes (9–12). The establishment of normal serum hepcidin values is necessary in order to define the cut-off points and characterize the measurement as normal or abnormal. There are limited data on normal hepcidin values in healthy adult population (13, 14). To our knowledge, there are many reports on classic iron parameters in healthy children (15–17) but there are very few studies

concerning hepcidin reference ranges in childhood. Most of them evaluate serum hepcidin levels in pathological conditions (18). The aim of our study was to evaluate the serum hepcidin levels in healthy children according to age and sex, and compare them with the well-established iron parameters.

The present prospective longitudinal study was conducted in a 3-year time period between 2008 and 2011. Procedures followed were in accordance with the Helsinki Declaration of 1975 as revised in 2008. The ethics committee of the Hospital as well as the Athens University Medical School approved the research protocol. Our study determines human hepcidin in children aged 2 to 12 years.

We included 180 children (88 boys, mean age: 67.55 ± 39.26 months, median: 60, range: 24 to 156 months) admitted to the First and Second Department of Pediatrics, University of Athens. The subjects belonged to urban Greek population and nationality, and participated voluntarily in the study. Informed parental consent was obtained in advance. After a thorough physical examination, parents filled in a detailed questionnaire, asking to include any history of anemia, chronic illness, or folic acid and/or iron dietary supplementation. Children under antibiotic treatment and with a medical history of a chronic disease, blood transfusion within the past 4 months, or other comorbidities were excluded from the study as well as children with hereditary or acquired anemia and hemochromatosis.

Taking into account that hepcidin is a hormone with diurnal variation (13, 14), we collected all blood samples at 08.00 a.m. after overnight fasting. In our stratification, serum hepcidin-25 isoform was detected by using a commercial competitive enzyme-linked immunosorbent assay (ELISA) kit (Hepcidin ELISA; DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions. The hepcidin ELISA is a solid-phase ELISA, based on the principle of competitive binding. The microtiter wells were coated with a monoclonal antibody directed toward the antigenic site of the bioactive hepcidin-25 molecule.

Endogenous hepcidin of a patient sample competes with the added hepcidin-biotin conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed off. Incubation with a streptavidin peroxidase enzyme complex and a second wash step follows. The addition of substrate solution results in a color development, which is stopped after a short incubation. The intensity of color developed is reverse proportional to the concentration of hepcidin in the patient sample. The minimum detectable concentration was 0.6 ng/ml while inter- and intraassay coefficients of variation were 7.37 and 4.1%, respectively. The hepcidin concentration samples were stored at -80°C for 5 to 8 months before analysis.

TABLE 1. Differences in Hepcidin, Ferritin, and Hb According to Age Group Stratified by Gender

	Mean ($\pm$ SD), median (range)	2–4.9 years ($n = 45$)	5–9.9 years ($n = 37$)	10–12 years ($n = 6$)	P^*
Boys	Hepcidin (ng/ml)	45.8 (± 23.95), 43.62 (10.32–115.73)	61.27 (± 26.04), 57.22 (6.78–118.86)	47.58 (± 18.94), 48.41 (16.58–74.57)	0.01
	Ferritin (ng/ml)	44.65 (± 31.24), 34.5 (11–173)	49.67 (± 30.74), 40 (17–135)	60.16 (± 40.5), 58.5 (19–128)	0.60
	Hb (gr/dl)	12.16 (± 1.02), 12.1 (9.8–13.9)	12.9 (± 0.95), 13 (10.3–15.1)	14.1 (± 1.3), 13.6 (13.4–16.9)	<0.001
	Hepcidin (ng/ml)	51.39 (± 28.74), 45.98 (4.16–137.83)	55.38 (± 18.18), 49.78 (18.97–101.58)	60.84 (± 31.63), 47.94 (20.52–107.12)	0.42
Girls		2–4.9 years ($n = 48$)	5–9.9 years ($n = 34$)	10–12 years ($n = 10$)	
	Hepcidin (ng/ml)	51.39 (± 28.74), 45.98 (4.16–137.83)	55.38 (± 18.18), 49.78 (18.97–101.58)	60.84 (± 31.63), 47.94 (20.52–107.12)	0.42
	Ferritin (ng/ml)	52.62 (± 33.05), 49(8–160)	47.44 (± 34.29), 38 (14–188)	38.9 (± 39.8), 26 (12–145)	0.10
	Hb (gr/dl)	12.04 (± 1.15), 12.05 (9.8–14.4)	12.9 (± 1.0), 12.9 (11.9–15.6)	12.79 (± 0.73), 12.8 (11.6–13.9)	0.002
P^{**}	Hepcidin (ng/ml)	0.3	0.3	0.66	
	Ferritin (ng/ml)	0.12	0.87	0.15	
	Hb (gr/dl)	0.7	0.7	0.01	

*Kruskal–Wallis test, **Mann–Whitney U test.

Hb, hemoglobin.

Other hematological parameters—hemoglobin (cut-off point of anemia was determined in hemoglobin concentration less than 11 gr/dl), ferritin, liver enzymes, cholesterol, creatinine, and C-reactive protein—were evaluated by the same laboratory, reassuring that all the subjects of the study had normal hepatic and renal function and were infection-free. Body mass indexes (BMI) of all subjects were within normal values (<85th percentile) for age and sex (15, 19). No restricted dietary habits were reported.

All statistical analyses and data management were performed using STATA for Windows, version 8.5, (Stata-Corp LP, College Station, TX, USA). Data are expressed as mean $\pm$ SD, median, and range. Groups were defined using age and sex classification. Three age groups were defined in the study population: age group 1 (2–4.9 years), age group 2 (5–9.9 years), and age group 3 (10–12 years). Data between groups were compared using non-parametric Mann–Whitney U test and Kruskal–Wallis test (independent samples), where applicable. Association between numerical variables was assessed with nonparametric Spearman's rho coefficient. $P < 0.05$ was considered significant.

Hepcidin distribution was slightly right-skewed, with Pearson's skewness index of 0.53 and Kurtosis index of 2.91. This was mainly due to the relatively small number of observations. Hepcidin distribution in all the children and according to gender, median value was 46.94 mg/ml in boys and 46.79 ng/ml in girls. No significant differences were observed in boys versus girls. There seem to be significantly higher values of hepcidin in older children (age group 1: 48.69 (± 26.54) ng/ml, age group 2: 58.45 (± 22.66) ng/ml, age group 3: 55.87 (± 27.64) ng/ml). This trend was constant and statistically significant in

boys after gender and age group stratification (Table 1). Although this trend was more prominent in girls, it was not statistically significant because of the small sample size.

Ferritin was found higher in older boys whereas it was found lower in older girls, although this trend was not statistically significant. Hemoglobin was significantly higher in older children, with significantly higher values in boys (Table 1). Hepcidin was found to be positively correlated with hemoglobin (Spearman's rho = 0.14, $P = 0.04$), but no correlation was found between hepcidin and ferritin (Spearman's rho = -0.05 , $P = 0.43$).

Although the significance of serum hepcidin measurement in clinical practice is well established in adults, there are very few data concerning its evaluation in pediatric population. On the other hand, the variety in measurement techniques of hepcidin did not offer the opportunity to define normal values. The aim of our study was to provide reference values for serum hepcidin concentrations in healthy children according to age and sex by using the most recent and reliable ELISA kit. To our knowledge, this is the second study that has used the same ELISA kit and addressed the specific issue in a large pediatric sample (180 children) that included children as young as 2 to 12 years old. Cangemi et al. reported serum hepcidin reference values, using the same commercial ELISA kit, in 86 healthy children, which served as control group for children with systemic juvenile idiopathic arthritis (16).

All of our children were evaluated in the morning after overnight fasting, in order to avoid the circadian alterations of serum hepcidin. We demonstrated that serum hepcidin concentrations were higher in older children (age groups 2 and 3, Table 1) compared to those in the younger

ones (group 1). We might suppose that younger children have increased need for iron due to their higher rate of growth, thus their serum hepcidin concentration is lower, increasing the amount of circulating iron. In Cangemi's paper, there was no age-related difference in serum hepcidin levels. That may be attributed to the smaller sample of study population ($n = 86$).

Evaluation of hepcidin concentration according to age and sex revealed a statistically significant difference in older boys (age groups 2 and 3, Table 1; $P = 0.01$). This is consistent with the higher growth rate of boys compared to girls reflecting the increased need for iron.

The same findings were more prominent in girls but were not statistically significant. One possible explanation could be the small sample size ($n = 10$). One could argue this is correct due to the increased iron needs in adolescent girls and their higher risk for iron deficiency. Due to the fact that none of our female subjects was iron-deficient based on serum ferritin measurements, a larger sample is needed to clarify the above observation. Another possible explanation could be the absence of menstruation in our study female group (group 3). Cangemi et al. found higher levels of serum hepcidin concentrations in prepubertal versus postpubertal females, even though the difference was not statistically significant. The difference between Cangemi's study and our results could be explained by the older females (81.4% below 15 years), which were enrolled in that study compared to our group (age group 3) where the oldest females were only 12 years old without menstruation (16). In another adult paper, Galesloot et al. reported that serum hepcidin concentrations were lower in premenopausal than postmenopausal women but were constant over age in men (14). In another cohort, serum hepcidin was found to be a useful indicator of deficient iron stores in premenopausal female blood donors (20).

Serum ferritin levels in our study population were not correlated with serum hepcidin concentrations. This is consistent with Cangemi's paper concerning pediatric population (16). On the contrary, correlation between hepcidin concentration and serum ferritin levels has been found in adults (14). There are at least two possible explanations for the above finding. The first could be the relatively small size of our study group. The second explanation could be a differentiation in iron metabolism between children and adults with additional unknown factors being involved in iron regulation between those two age groups.

Interestingly, we noticed that our median serum hepcidin values in both sexes are significantly higher compared with the adult measurements, but were consistent with measurements in the Cangemi's study (14, 16). The comparison was made with studies that used the same ELISA kit (DRG Hepcidin ELISA kit). This finding could reflect the alterations in iron metabolism in adults due to

different needs and the possible presence of other comorbidities and justifies our aim in setting up reference values for serum hepcidin in healthy children.

In conclusion, our study provides data on normal range values of serum hepcidin in healthy children. The use of a reliable commercial kit reassures the reproducibility of the results. Due to the lack of bibliographic references concerning the pediatric population, our results could be used along with larger studies to establish normal serum hepcidin values in different age groups, providing a useful tool in evaluating iron metabolism parameters between healthy children and children with inflammatory diseases or iron metabolism disturbances.

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